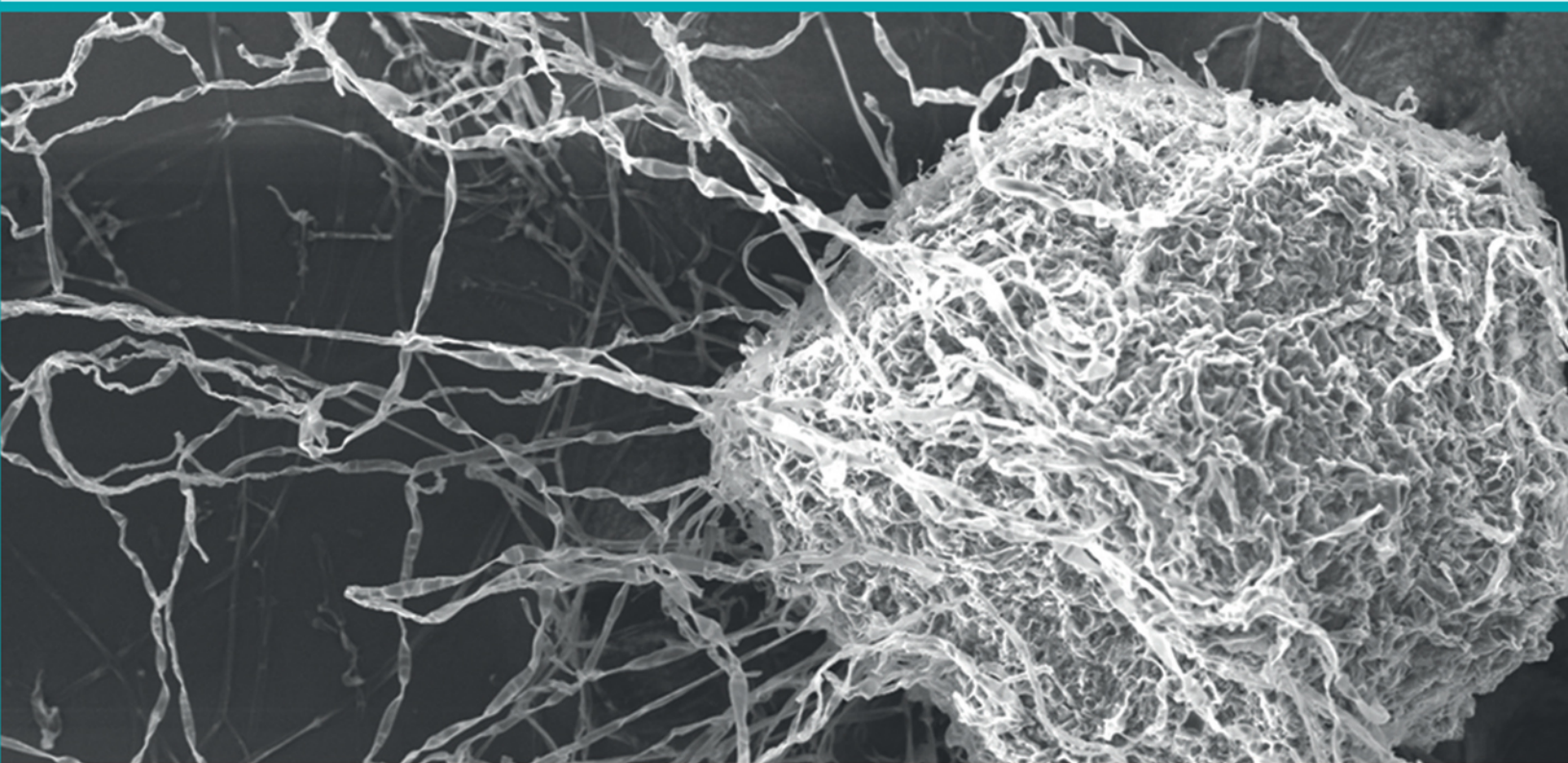


Edited by
Vivek Sharma
Richa Salwan
Laith Khalil Tawfeeq Al-Ani

MOLECULAR ASPECTS OF PLANT BENEFICIAL MICROBES IN AGRICULTURE



Molecular Aspects of Plant Beneficial Microbes in Agriculture

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Molecular Aspects of Plant Beneficial Microbes in Agriculture

Edited by

Vivek Sharma

University Centre for Research and Development, Chandigarh University,
Gharuan, India

Richa Salwan

College of Horticulture and Forestry (Dr. Y. S. Parmar University
of Horticulture & Forestry) Neri, Hamirpur, India

Laith Khalil Tawfeeq Al-Ani

Department of plant protection, College of Agriculture,
University of Baghdad, Baghdad, Iraq
School of Biology Science, Universiti Sains Malaysia,
11800 Minden, Pulau Pinang, Malaysia



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List of contributors

- Liliana Aguilar-Marcelino** Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Mexico
- Mushtaq Ahmed** Department of Environmental Sciences, School of Earth and Environmental Sciences, Central University of Himachal Pradesh, Kangra, India
- Laith Khalil Tawfeeq Al-Ani** Department of Plant Protection, College of Agriculture, University of Baghdad, Baghdad, Iraq; School of Biology Science, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia
- Anshu Alok** University Institute of Engineering & Technology, Panjab University, Chandigarh, India; National Agri-Food Biotechnology Institute, Government of India, Mohali, India
- S. Aravindan** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- Mathew S. Baite** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- Raina Bajpai** Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India
- Despoina Beris** Laboratory of Virology, Department of Phytopathology, Benaki Phytopathological Institute, S. Delta 8, 14561 Kifissia, Athens, Greece
- Hareram Birla** Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India
- Didier Bogusz** UMR DIADE, Rhizogenèse, Institut de Recherche pour le Développement (IRD), Montpellier, France
- Valquiria Broll** Laboratory of Bioinorganic Chemistry, Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy
- Lorena Carro** Microbiology and Genetic Department. University of Salamanca. Salamanca. Spain
- Antony Champion** DIADE UMR 232 IRD/CIRAD/ University of Montpellier, Montpellier, France
- Wenfeng Chen** State Key Laboratory of Agrobiotechnology, Beijing, P.R. China; College of Biological Sciences and Rhizobium Research Center, China Agricultural University, Beijing, P.R. China
- Wenxin Chen** State Key Laboratory of Agrobiotechnology, Beijing, P.R. China; College of Biological Sciences and Rhizobium Research Center, China Agricultural University, Beijing, P.R. China
- Juliana Oliveira de Carvalho** Graduate Program in Cell and Molecular Biology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
- Filippe Elias de Freitas Soares** Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil; Departamento de Química, Universidade Federal de Lavras, Minas Gerais, Brazil
- Nasser Delangiz** Department of Plant Biotechnology and Breeding, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
- Nathalie Diagne** Institut Sénégalais de Recherches Agricoles/ Centre National de Recherches Agronomiques (ISRA/CNRA), Bambey, Senegal.; LMI LAPSE, Centre de Recherche de Bel Air, Dakar, Senegal.
- Hagera Dilmashin** Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India
- Pape Ibrahima Djighaly** LMI LAPSE, Centre de Recherche de Bel Air, Dakar, Senegal; LCM, IRD/ ISRA, UCAD, Centre de Recherche de Bel Air, Dakar, Senegal; Département d'Agroforesterie, Université Assane Seck, Ziguinchor, Senegal
- Éverton Kort Kamp Fernandes** Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil
- Claudine Franche** UMR DIADE, Rhizogenèse, Institut de Recherche pour le Développement (IRD), Montpellier, France
- Edson Luiz Furtado** Department of Plant Production, College of Agronomic Science Fazenda Experimental Lageado, São Paulo State University, São Paulo, Brazil

- Geetika** Department of Environmental Sciences, School of Earth and Environmental Sciences, Central University of Himachal Pradesh, Kangra, India
- Mansour Ghorbanpour** Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran
- Patrícia Silva Gôlo** Departamento de Parasitologia Animal, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil.
- Olga Gómez-Rodríguez** Fitopatología, Colegio de Postgraduados, Texcoco, Mexico
- Oscar González-López** Research Group in Engineering and Sustainable Agriculture, Department of Engineering and Agricultural Sciences, Environment Institute Natural Resources and Biodiversity, University of León, León, Spain
- A.B.M. Gulzar** Department of Biotechnology, Assam University, Silchar, India
- Chen Guo** College of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, P.R. China
- Vipin Hallan** Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, India.
- Trinh Xuan Hoat** Plant Protection Research Institute (PPRI), Vietnam Academy of Agricultural Sciences (VAAS), Ha Noi, Vietnam
- Valérie Hocher** LSTM, UMR 040 IRD/INRA/CIRAD, University of Montpellier/SUPAGRO, Montpellier, France.
- Ikbāl** Department of Molecular Biology Biotechnology Bioinformatics, College of Basic sciences, CCS-Haryana Agricultural University, Hisar, India
- Siva Satya Mohan Jampala** Post-Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India
- P. Jeyakumar** Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore, India
- Yogeshvari K. Jhala** Department of Agricultural Microbiology, B. A. College of Agriculture, Anand Agricultural University, Anand, India
- Gurdeep Kaur** Department of Agricultural Sciences, Chandigarh University, Mohali, India
- Jaspreet Kaur** University Institute of Engineering & Technology, Panjab University, Chandigarh, India
- Solmaz Kazemalilou** Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
- U. Keerthana** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- Chetan Keswani** Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India
- Ekta Khare** Department of Microbiology, Chhatrapati Shahu Ji Maharaj University, Kanpur, India
- Surender Kumar** Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, India; Institute of Plant Protection, ARO, The Volcani Center, Rishon LeTsiyon, Israel
- Indu Kumari** Department of Zoology, Career Point University, Hamirpur, India
- Nidhi Kumari** Division of Crop Protection, ICAR-Central Institute for Subtropical Horticulture, Lucknow, India
- Reenu Kumari** Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, India; Institute of Plant Protection, ARO, The Volcani Center, Rishon LeTsiyon, Israel
- Behnam Asgari Lajayer** Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
- Erik Limpens** Laboratory of Molecular Biology, Wageningen University & Research, Wageningen, The Netherlands
- Fernanda Cortez Lopes** Graduate Program in Cell and Molecular Biology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; Center for Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
- María Eugenia López-Arellano** Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Mexico
- Rita Mahapatra** Post-Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India
- Anudeep B. Malannavar** Department of Plant Pathology, CSK HPKV, Palampur, India
- Anne Helene Souza Martinelli** Department of Biophysics & Department of Molecular Biology and Biotechnology, Biosciences Institute (IB), Federal University of Rio Grande do Sul, Porto Alegre, Brazil
- P.B. Mazumder** Department of Biotechnology, Assam University, Silchar, India
- Pedro Mendoza-de-Gives** Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Mexico

- Esther Menéndez ICAAM** - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Laboratório de Microbiologia do Solo, University of Évora, Évora, Portugal
- Ewa B. Moliszewska** Faculty of Environmental and Technical Sciences, Institute of Environmental Engineering and Biotechnology, Opole University, Opole, Poland
- Małgorzata Nabrdalik** Faculty of Environmental and Technical Sciences, Institute of Environmental Engineering and Biotechnology, Opole University, Opole, Poland
- Mariama Ngom** LMI LAPSE, Centre de Recherche de Bel Air, Dakar, Senegal.; LCM, IRD/ISRA, UCAD, Centre de Recherche de Bel Air, Dakar, Senegal.
- Deepak G. Panpatte** Department of Agricultural Microbiology, B. A. College of Agriculture, Anand Agricultural University, Anand, India
- Nishat Passricha** Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
- Jai Singh Patel** Dalhousie University, Department of Plant food and Environmental Sciences, Nova Scotia, Canada
- Zalak M. Patel** Post-Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India
- Kishor Sureshbhai Patil** Division of Biotechnology, Chonbuk National University, Iksan, Republic of Korea
- Shanshan Peng** College of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, P.R. China
- Céline Pesce** University of New Hampshire, Durham, NH, United States
- S.R. Prabhukarthikeyan** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- S. Raghu** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- Md. Mahtab Rashid** Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India.
- P.C. Rath** Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India
- Waseem Raza** Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing, China
- David Emmanuel Reyes-Guerrero** Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Mexico
- David Ruano-Rosa** Woody and Horticultural Crops Unit (Agricultural Research Area), Technological Agricultural Institute of Castilla y León, Valladolid, Spain
- Davood Saghafi** Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
- Shabnam K. Saifi** Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
- Richa Salwan** College of Horticulture and Forestry (Dr. Y. S. Parmar University of Horticulture & Forestry) Neri, Hamirpur, India
- Yimin Shang** College of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, P.R. China
- Anu Sharma** University Centre for Research and Development, Chandigarh University, Sahibzada Ajit Singh Nagar, India
- Shikha Sharma** Department of Environmental Sciences, School of Earth and Environmental Sciences, Central University of Himachal Pradesh, Kangra, India
- Vivek Sharma** University Centre for Research and Development, Chandigarh University, Sahibzada Ajit Singh Nagar, India
- Qirong Shen** Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing, China
- Virendra K Sikka** Department of Molecular Biology Biotechnology Bioinformatics, College of Basic sciences, CCS-Haryana Agricultural University, Hisar, India
- H.B. Singh** Department of Mycology and Plant Pathology Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India
- S.P. Singh** Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India
- Bijoya Singha** Department of Biotechnology, Assam University, Silchar, India
- S. Srividhya** Division of Field Crop Improvement and Protection, ICAR- Central Island Agricultural Research Institute, Port Blair, India
- R. Surendranath** Department of Horticulture and Sericulture, Hyderabad, India

Sergio Svistoonoff LSTM, UMR 040 IRD/INRA/CIRAD, University of Montpellier/SUPAGRO, Montpellier, France.

Basavaraj Teli Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India.

Louis S. Tisa University of New Hampshire, Durham, NH, United States

Siddharth Tiwari National Agri-Food Biotechnology Institute, Government of India, Mohali, India

Narendra Tuteja Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

Swati Tyagi Genomics Division, National Academy of Agriculture Science, Rural Development Administration, Jeonju, Republic of Korea

Jelle van Creijl Laboratory of Molecular Biology, Wageningen University & Research, Wageningen, The Netherlands

Udaya Kumar Vandana Department of Biotechnology, Assam University, Silchar, India

Mohammad Behrouzi Varjovi Department of Agronomy and Plant Breeding, Faculty of Agricultural

and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran.

Nikon Vassilakos Laboratory of Virology, Department of Phytopathology, Benaki Phytopathological Institute, S. Delta 8, 14561 Kifissia, Athens, Greece

Edgar Villar-Luna CONACYT-Instituto Politécnico Nacional, CIIDIR-IPN, Unidad Michoacán, Jiquilpan, Mexico.

Rajababu V. Vyas Department of Agricultural Microbiology, B. A. College of Agriculture, Anand Agricultural University, Anand, India

Peng Wang Laboratory of Molecular Biology, Wageningen University & Research, Wageningen, The Netherlands

Manoj K. Yadav Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India

Sudheer Kumar Yadav Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, India.

Junjie Zhang College of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, P.R. China; Collaborative Innovation Center for Food Production and Safety of Henan Province, Zhengzhou, P.R. China

Chapter 1

Overview and challenges in the implementation of plant beneficial microbes

Vivek Sharma¹, Anu Sharma¹ and Richa Salwan²

¹University Centre for Research and Development, Chandigarh University, Sahibzada Ajit Singh Nagar, India, ²College of Horticulture and Forestry (Dr. Y. S. Parmar University of Horticulture & Forestry) Neri, Hamirpur, India

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1.1 Introduction

At present, agricultural farming uses 75% of total fresh water and that usage can be alarming in the coming days, if inclinations in population size and contemporary methods of farming continue (Molden et al., 2007). However, emphasis to enhance the food production to cope up with growing worldwide food requirements for perpetually growing human population generates severe ecological vulnerabilities, such as deficiency of water and salinity (Golladack et al., 2014) and extensive usage of chemicals in the form of fertilizers and pesticides. These parameters have intensified and impose main threats to our ecosystem and agricultural production (Trenberth et al., 2014; Lakshmanan et al., 2017). Plant beneficial microorganisms are an attractive alternative for sustainable and enhanced growth (Chaparro et al., 2013). The use of beneficial microbes in agriculture was largely underutilized in 18th century, however, U.S. Department of Agriculture endorsed the inoculation rhizobium for legume crops, after the experimental demonstration of their role in fixing nitrogen (Schneider, 1892). In last few years, a number of individual microbes have been explored for their role in plant growth promotion, N & P uptake (Afzal and Bano, 2008), and combating disease resistance (Harman et al., 2004; Sharma et al., 2017a,b; Sharma, et al., 2018a,b). Mostly, these efforts were limited to individual microbial strains (Sharma et al., 2012; Sharma et al., 2016a,b; Busby et al., 2017; Salwan et al., 2019).

Beneficial microorganisms can help in nutrient acquisitions such nitrogen from atmosphere, irons and from soil inhibit plant pathogens, and secrete metabolites which can augment the plant growth (Singh et al., 2011). These microorganisms represent comprehensive range of microorganisms including nitrogen-fixing bacteria both as symbiotic (Mendes et al., 2013) or free living, plant growth-promoting rhizobacteria (PGPR), and fungi involve in mycorrhizal associations (Mendes et al., 2013; Arora et al., 2016), as well as biocontrol agents (Sharma et al., 2012; Sharma et al., 2016a,b; Sharma et al., 2017a,b; Sharma et al., 2018a,b). Different members of bacteria including *Azotobacter*, *Azospirillum*, *Bacillus* spp, *Pseudomonas fluorescence*, *Rhizobium*, *Frankia* and other members of actinobacteria are

termed as PGPR, due to their growth promoting effect the plants (Nadeem et al., 2014). The plant beneficial fungal counterpart such as *Trichoderma* strains (Sharma et al., 2012; Sharma et al., 2016a,b; Sharma et al., 2017a,b; Sharma et al., 2018a,b) also suppress the pathogens their antagonistic activity by producing lytic enzymes (Sharma et al., 2012; Sharma & Shanmugam, 2012, Sharma et al., 2016a,b, 2017a,b; Sharma et al., 2018a,b) and secondary metabolites, promotes the plant growth through growth regulator production (Bhattacharyya and Jha, 2012) and enhance the plant immunity. The other benefits of these plants beneficial microorganisms involves increase in the formation of secondary roots, roots elongation, assisting nutrient acquisition, and suppression of the plant pathogens (Ali et al., 2014; Bashan et al., 2014; Arora et al., 2016). The other benefits competition for space, antibiotic production, symbiotic association in soil (Kloepper et al., 1980; Kilian et al., 2000; Avis et al., 2008) or phylloplane (Araujo et al., 2005; Raaijmakers and Mazzola, 2016; Arora et al., 2016) and even alleviate salt stress tolerance in crops (Egamberdieva et al., 2013; Nadeem et al., 2014). The role of microbiomes to augment crop production is prehistoric and evidences can be found back to \$300 BCE (Vessey, 2003). The first industrial bioinoculant, 'nitrogin', patented during the microbiology golden age in 1896 and past the Haber–Bosch method by 15 years (Sahoo et al., 2013). Currently, the Organic Materials Review Institute (OMRI) (<https://www.omri.org/omri-lists>) lists over 170 'microbial inoculants' and more than 274 as 'microbial products', for crop fertilizers or crop management (Finkel et al., 2017).

Nearly 45 million hectares of worldwide irrigated area is saline which limit the plant growth and hence decreases crop production (Munns and Tester, 2008). The effect of plant beneficial microorganisms against salinity is well documented. For example, *Bacillus amyloliquefaciens* a plant growth promoting rhizobacteria, improve the plant growth in rice (Nautiyal et al., 2013). Similar to this, inoculation of *Azotobacter chroococcum*, enhance the plant biomass in maize under salt stress (Rojas-Tapias et al., 2012). The combination of rhizobia and *Pseudomonas* spp. improved negative effect of salt stress in *Galega officinalis* plants (Egamberdieva et al., 2013; Arora et al., 2016). On the other side, biocontrol agents are gaining attentions for plant diseases and pest managements and offers resilience to biotic and abiotic stresses (Mendes et al., 2013; Weller et al., 2002). Four categories of biocontrol systems; i- natural, ii-conservation, iii-classical, and iv-augmentative biological control are defined in the literature (Eilenberg et al., 2001; Cock et al., 2010). In natural biocontrol works without human involvement and here, pest/pathogens are managed using naturally prevailing beneficial organisms (Waage and Greathead, 1988). The second conservation based biocontrol system, involves human actions which defend and arouse the performance of prevailing natural enemy. In third category, natural enemies collected from one area are released in pest invasive area (Cock et al., 2010). Since it the first type of biocontrol system commonly practiced, it is known as 'classical' biocontrol system (DeBach, 1964). In fourth biocontrol system, natural enemies such as parasitoid, predator or microorganism are mass-multiplied and then used to manage the pests/pathogens in crops (Cock et al., 2010; Lorito et al., 2010; van Lenteren, 2012; Parnell et al., 2016; van Lenteren et al., 2018). The augmented control system includes use of *Bacillus*, *Azospirillum*, *Pseudomonas*, *Rhizobium*, *Beauveria*, *Trichoderma*, *Metarhizium*, and actinomycetes of the genus *Streptomyces*.

Other category of plant beneficial microbes includes "mycorrhiza" represents symbiotically associated soil fungi with the roots of plants (Bonfante and Genre, 2008). Mycorrhiza are involved in nutrients uptake and provide protection to plants from other stress factors. These fungi are divided into two subtypes; i-ectomycorrhizae and ii-endomycorrhizae. In endomycorrhizae, the hyphae infiltrate the root tissue and develop an intracellular symbiotic association with the host (Bonfante and Anca, 2009). Arbuscular mycorrhiza (AMF) have pronounced impact in tropical ecosystem. It includes fungi that are one of most frequent in the roots of crop plants (Bonfante and Genre, 2008). AMF are obligate symbionts which belong to the Glomeromycota phylum; these and have generous impact on crop production (Redecker et al., 2013; Arora et al., 2016). The mycorrhizal fungi, root modulated legume bacteria and beneficial microorganisms such as *Azotobacter chroococcum* or *Bacillus megaterium* recognized as root symbionts in 1950s were used to treat seeds of agricultural crops were treated with bacteria such as for improving plant growth and yield (Brown, 1974). In late 20th century, several strains of *Pseudomonas* and *Azospirillum* were also described (Jacoby et al., 2017).

Interestingly the demand for developing environmental friendly agricultural technologies which do not have adverse effects on our ecosystem, are reachable to the farmers, and helpful to the farmers and can improve food productivity are an attractive choice. Here, in this chapter a brief of different plant beneficial microorganisms is described. We will discuss plant–microbe associations, their manipulation for increasing the plant productivity in agronomic location. Emphasis is also given to discuss tools having potential to ameliorate plant stresses in view the basic of cost-effective, societal, and ecofriendly methods (Ray and Craven, 2016; Lakshmanan et al., 2017).

1.2 Soil microbiome and its significance

The rhizospheric soil near to roots can consist of 10^{11} microbe per gram of root tissue (Rolli et al., 2015) and more than thirty thousands of prokaryotic species (Ngumbi and Kloepper, 2016). Despite that the difficulties of characterizing

microbiome that is not unculturable is another concern. overall, microbial communities, above and below the ground are distinct, and differs in taxonomic assortment, structural organization, and function (Wagner et al., 2016). The advancements in molecular tools such as high-throughput next-generation DNA sequencing, played vital role in characterizing the root microbiomes allied with agronomically important crops including rice (Edwards et al., 2015), maize (Peiffer et al., 2013), sweet potato (Marques et al., 2014), sugarcane (Yeoh et al., 2015), banana (Köberl et al., 2015), barley (Bulgarelli et al., 2015), the model plant, *Arabidopsis thaliana* (Lundberg et al., 2012; Bulgarelli et al., 2015) and grasslands (Zhang et al., 2016) in the last decade (Lakshmanan et al., 2017). The use of high throughput systems such as 454 Genome Sequencer (Roche), HiSeq 2500 (Illumina), Nanopore (Oxford Nanopore, Technologies) AB SOLiD System (Life Technologies), coupled to statistical tools, have played vital role in describing the soil microbial communities with unparalleled accuracy and depth (Caporaso et al., 2012).

In the early 1900s, at University of California at Riverside, USA, researchers coined the word ‘biological control’, and shared the environmental, economic, and other societal benefits of it (van Lenteren et al., 2006; van Driesche et al., 2010; De Clercq et al., 2011). Nevertheless, since that time and fantasies of ladybird, things have changed massively (Warner et al., 2011), lots have changed. Presently, the market potential of biofertilizers and biopesticides are determined by number of parameters including crop type, active ingredients, applications, and even geographical conditions. The worldwide market of pesticides up to the year 2014 was about US \$1.72 billion which is anticipated to touch US \$4.17 billion mark by 2023, at a yearly growth rate of 9.9%, in of the years between 2015 and 2023 (Markets and markets, 2014). The biofertilizer market segment is estimated to touch USD 1.66 billion mark by 2022 at annual growth rate of 13.2% between 2015 and 2022, presently represents approximately 5% of the overall chemical based fertilizers (BCC Research, 2014). Worldwide, the biofertilizer share is ruled by nitrogen-fixing microorganisms such as *Rhizobium* spp., *Azotobacter* spp., *Actinorhizobium* spp., *Frankia*, blue green algae *Azospirillum* spp., and others. beside their use for leguminous crops, they are also explored for other crops such as paddy and sugarcane fields.

Plant growth rhizobacteria (PGPR) categorised them into biofertilizers and biocontrol agents or biopesticides (Glick, 2012). The former increases plant growth via by accelerating processes in the soil which leads to enhanced nutrients availability in a form which can be easily assimilated by plants. This group includes N₂ fixing, phosphate solubilizing and mobilizing or iron mobilizing biofertilizers. The second group which includes biocontrol agents (BCA) or biopesticides main function is suppressing or controlling plant disease. They can be categorized as bacteria per se or as compounds derived from bacteria acting as biocontrol agents (Timmusk et al., 2017). The plant beneficial microbial population here may represent PGPR, biocontrol agents, N₂-fixing blue green algae, mycorrhiza, endophytes and other members. Two types of rhizobacteria; extracellular PGPR and intracellular PGPR has been identified by Gray and Smith (2005) (Fig. 1.1). The first class group occurs in the rhizosphere and in apoplastic space, and on the rhizoplane whereas the second group thrive inside the root cells and there constitute special nodular structure e.g. rhizobia and *Frankia* (Qin et al., 2016). Microbes such as *Glomus versiforme* contains transporters for inorganic phosphate (Pi) which are involved in intake of phosphate directly from the soil. Other strain *G. intraradice* contains glutamine synthase transcript which enhances the nitrogen digestion in fungi that can be elated at later stage to the host plant (Salvioli et al., 2012). A diffusible compound known as “Myc factors” is found to induce the expression of symbiotic-specific *MtENOD11* genes in *Medicago truncatula* roots (Kosuta et al., 2003). It is similar to “Nod factors” released as signaling molecules by *Mesorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium* collectively known as

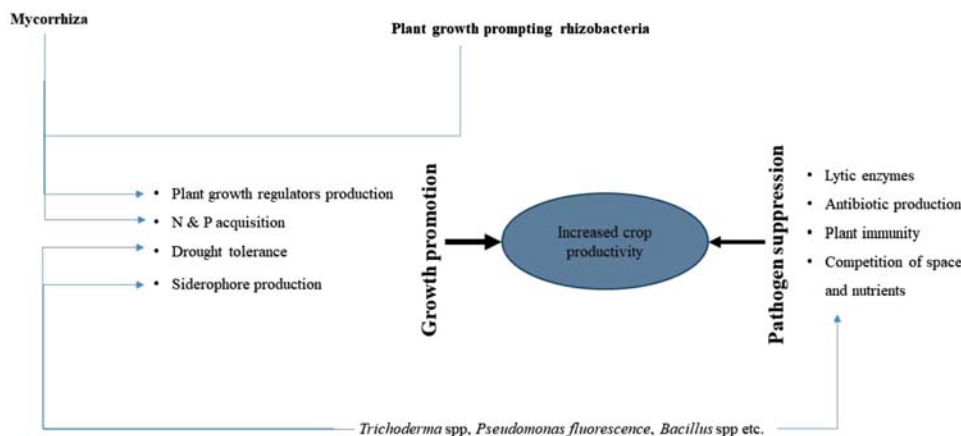


FIGURE 1.1 Role of plant beneficial microorganisms in crop productivity. Different plant beneficial microorganisms’ either directly benefit the host plant through different growth regulators productions and nutrient acquisitions or indirectly by their antagonistic and plant immune system modulations activity enhances the crop yields.

Rhizobia (Geurts and Bisseling, 2002). At present, diversity of plant associated microbiomes is comparatively well characterized and even their functional aspects are now better understood.

The nod factor under is another diffusible compounds, activates the symbiosis (SYM) signaling pathway of host roots (Kosuta et al., 2003; Roberts et al., 2013) under nitrogen limiting conditions and helps the rhizobium to forms endosymbiotic relationship with legume plants. The mycorrhiza and rhizobia, share similarity with each other as secondary messenger Ca^{2+} is central to both (Sieberer et al., 2009). The microarray based experimental studies revealed that *Rhizobium leguminosarum* biovar *viciae*, upregulate different genes in pea, sugar beet and alfalfa plants. The secretion of indole acetic acid (IAA) by PGPR, prompts the nitric oxide (NO) production, which also play the role of secondary messenger and trigger a signaling pathways linked to plant growth and other developing processes in plant such as canola plants (Molina-Favero et al., 2007; Bhattacharyya and Jha, 2012; Bhardwaj et al., 2014). The hydrolysis of 1-aminocyclopropane-1-carboxylate (ACC), an ethylene precursor by ACC-deaminase activity is other mechanism which makes favorable conditions for plant growth. ACC-deaminase activity has been reported for *Bacillus pumilus*, *Pseudomonas putida* and other PGPR (Bhattacharyya and Jha, 2012; Bhardwaj et al., 2014). In non-leguminous plants such as rice, *Sinorhizobium meliloti*, a rhizobium species, enhance growth by the regulation of plant hormone level endogenously confer tolerance to stress (Chi et al., 2010).

1.3 Plant microbiome and its potential

The marketing of plant beneficial microorganisms are of vital significance in agricultural fields due to their environmental safety, sustainability and multiple benefits to the host plants as discussed earlier for better nutrients acquisition, enhanced plant growth and tolerance to environmental stress factors (Sharma et al., 2017a,b). The plant associated microbiome, gained the attention, due to tie multiple benefits to the host plants. The plant microbiome comprises the different useful gene pool of prokaryotic and eukaryotic origin which is linked with the habitats of host plant. The microbiome in plant rhizosphere works as extremely evolved exterior active milieu for plants (For more detail information see Bais et al., 2006; Badri et al., 2009; Pineda et al., 2010; Philippot et al., 2013; Rout and Southworth, 2013; Spence et al., 2014). In other words, it represents the second genome of the plant (Berendsen et al., 2012) and reveal positive or negative effect on plant productivity, immunity and yield (Lakshmanan et al., 2014). The co-inoculation of PGPR or AMF, found to enhance the efficient use of fertilizers. For example, the combined use of PGPR and AMF was found, better suited to for N and P uptake compared to fertilizers alone (Adesemoye et al., 2009). Different plant beneficial microorganisms such as *Azotobacter*, *Azospirillum*, *Bacillus* spp, *Pseudomonas*, *Rhizobium*, blue green algae and other PGPRs, deliver substantial quantity of N for the plant growth and crop productivity (Choudhury and Kennedy, 2004; Dhanasekar and Dhandapani, 2012; Bhardwaj et al., 2014).

The inoculation of *Rhizobium trifolii* in *Trifolium alexandrinum* plants, led to high number of nodulation and higher biomass under saline stress (Hussain et al., 2002; Antoun and Prevost, 2005). Similar to this, the inoculation of *P. fluorescens* MSP-393, led to the production of osmolytes and salt-stress induced proteins which help the plants under salinity (Paul and Nair, 2008). Also, the inoculation of *P. putida* Rs-198 strain enhanced seed germination and plant growth under high salt and alkaline conditions by upregulating the transport of K^+ , Ca^{2+} and Mg^{2+} and by declining the Na^+ absorption (Yao et al., 2010). In a few cases, *Pseudomonas* strains also imparted systemic response in *Arabidopsis thaliana* against *P. syringae* by 2,4-diacetylphloroglucinol (DAPG) (Schnider-Keel et al., 2000; Weller et al., 2012). The production of calcisolin via *P. alcaligenes* PsA15 and *Bacillus polymyxa*, imparts tolerance abiotic stresses such as high temperature and saline conditions (Egamberdiyeva, 2007). The inoculation of AM fungi also increases plant growth under saline conditions. In other case, inoculation of *Achromobacter piechaudii* strains enhances the tomato and pepper plants biomass under water stress and NaCl concentration level of 172 mM (Alavi et al., 2013). The root endosymbiont fungi *Piriformospora indica*, induced defense response in host plants against saline stress. Moreover, *Azotobacter* in addition to nitrogen fixation, secreted thiamine and riboflavin, plant growth regulators including IAA, gibberellin (GA) and cytokinin.

The arbuscular mycorrhizal fungi belonging to the subphylum Glomeromycotina, are obligate symbionts in more than >60% of terrestrial plant species (van der Heijden et al., 2015; Spatafora et al., 2016). These fungi play vital role in the nutrients acquisition (Smith and Read, 2008), enhancing the resistance to pathogens (Vigo et al., 2000) and/or tolerance to water scarcity and osmotic stress in the associated host plants (Augé et al., 2015). The mycelia of mycorrhizal member acquire ammonium and nitrate from nearby soil and then afterward, these molecules are obtained by mantle and Hartig net, from there they are supplied to the associated plant. The mycorrhiza orchestrated N uptake is predominant in organic agricultural fields or under drought (Hodge and Storer, 2015; Bukovská et al., 2018; Řezáčová et al., 2018a,b). In exchange of N and P, the fungus receive food in the form of simple carbohydrates and/or fatty acids

(Roth and Paszkowski, 2017) and its amount varies from 0.9 to 20%, of host plant gross photosynthesis rate (Konvalinková et al., 2017; Slavíková et al., 2017). However, the precise mechanism responsible for nutrient flow, still not precisely discovered (Bücking et al., 2016).

1.4 Methods to investigate the composition of root exudate

The plant root exudates are one of the major driving force for rhizospheric interactions amid microorganisms, plants, and insects (Bais et al., 2006). Therefore, it is of paramount importance to understand the role of root exudate in determining the microbial community profile for plant benefits. A number of methods have been defined for analyzing of the plant root exudate compositions. In one method, the exudates of mature trees were collected (Phillips et al., 2008). However, since this method lacks sterile conditions therefore, microbial populations can lead to major impact. The other method uses flavonoid glucosides degradation under two types of soils for legume cultivations (Carlsen et al., 2012). In addition, the other alternate methods based axenic hydroponic systems have been used (Badri et al., 2008; Oburger et al., 2013; Strehmel et al., 2014). These methods are easy to control. However hydroponic systems are artificial in nature and may represent stress response due to oxygen restraint and lack of proper root support. Still, hydroponics systems are better for sampling of exudates. In a study, on *Arabidopsis thaliana* root exudates, by LC-MS analysis, discovered that majority biomolecules are produced late (Badri et al., 2008). Several other factors, such as host plant genotype, type of soil, and methods of cultivation, play vital role in determining the microbial community structure (Philippot et al., 2013; Turner et al., 2013; Huang et al., 2014). Using a stable isotope probing (SIP) method, Haichar et al. (2008) discovered that root exudates of different plants such as maize, wheat, rape, and *Medicago truncatula* grown under similar conditions play vital role in determining the bacterial community.

1.5 Diversity of the inoculum

The integration of plant beneficial microorganisms has emerged as an attractive method for combating biotic and abiotic stresses in plants. Keeping into the consideration of sustainability in agricultural, there is need to explore methods and practices which do not cause negative effects on our ecosystem, and are easily accessible to the farmers, and efficient in improving food productivity. The plant microbiome manipulation so far is largely restricted to selected bacteria or fungi that have a substantial influence on nutrient acquisition or plant health (Bhattacharyya and Jha, 2012), and finally increasing the crop productivity (Vessey, 2003; Lugtenberg and Kamilova, 2009) by alleviating abiotic or biotic stresses. Collectively mentioned as the plant microbiome, these microorganisms are second genome to the associated plant which offers unused and encouraging effects on plant growth, health and crop productivity (Berendsen et al., 2012). The microbial genome possesses comprehensive and extensive functional gene pool (Rout and Southworth, 2013). Even though mostly still a black box, the microbial diversity, species richness and its assortment in plant and soil sphere has been recognized in the model plants such as *Arabidopsis* (Bulgarelli et al., 2012a,b; Lundberg et al., 2012; Bai et al., 2015; Lebeis et al., 2015), and a few crop plants (Peiffer et al., 2013; Bulgarelli et al., 2015; Edwards et al., 2015). The application of advanced molecular approaches based on high-throughput DNA sequencing has significantly improved our present understanding of the microbiota residing in plant rhizosphere (Lakshmanan et al., 2017).

A mixture of inoculum is found to deliver the better plant disease resistance (Niu et al., 2015; Santhanam et al., 2015; Wei et al., 2015; Agler et al., 2016; Yasmin et al., 2016; Hu et al., 2016) and promotion of growth (Baas et al., 2016; Timm et al., 2016), than single strains. However, in some cases, consortium was equivalent, or less effective than, individual strains as confirmed by grapevines growth under water deficiency (Rolli et al., 2015). The consortium can be composed of related strains (Hu et al., 2016; Hu et al., 2016), or remotely related strains (Timm et al., 2016). In some examples, however, collective strain richness, of the biocontrol agents such as *Pseudomonas fluorescens*, resulted antagonism within followed by loss of plant defense (Becker et al., 2012). A combination of different species also led to constructing interactions between microorganisms. e.g., the combined inoculum of bacteria and AMF, enhances elongation of germ tube and branching of hyphae in AMF, and finally encouraged the symbiotic progress of the AMF in potato plants (Loján et al., 2016; Finkel et al., 2017).

1.6 Role of omic technologies and modeling tools in plant microbiome

A detailed understanding of microbiome assembly in plant rhizosphere will contribute in depicting organization of core microbiome and enable the fabrication of microbial communities for agricultural (Qin et al., 2016). The microbiome structure is determined by plant–microbe and microbe–microbe and microbe–plant–microbe communications

(Agler et al., 2016). The availability of space and nutrients also influence the microbial community structure (Kroll et al., 2017). The continuous growth in large scale data in biology for genome, transcripts, proteins and secondary metabolites and data analysis tools is rapidly powering our understanding of microbiome research. The use of cutting edge omics tools have assisted us to obtain deep understandings into the organization and function of plant-microbiomes. The large scale genome sequencing of different plants and their associated microorganisms have played major in mining the underlying mechanisms of how host–microbe and microbe–microbe interactions help in niche establishment, and then contribute to colonization? (Kroll et al., 2017). Further, integrated and coordinated efforts will accelerate the incorporation of plant beneficial microbiome into agricultural practices. In several studies, plant microbiome organization and its function has been studied in model plant *Arabidopsis thaliana* (Delmotte et al., 2009; Bulgarelli et al., 2012a,b; Lundberg et al., 2012; Schlaeppi et al., 2014) as well in agricultural crops such as barley (Bulgarelli et al., 2015), soybean (Delmotte et al., 2009; Mendes et al., 2014; Rascovan et al., 2016), corn (Aira et al., 2010), wheat (Donn et al., 2015; Robinson et al., 2015), rice (Knief et al., 2012; Lucas et al., 2013; Edwards et al., 2015), and cottonwood trees (Shakya et al., 2013; Busby et al., 2017). These tools also helped in unraveling the physiological and molecular factors involved in plant root–microbe interactions and how these microbes can help developing stress tolerance against different factors such as salt and drought. Plant-associated microbiome are one of the key members during interaction with the associated host plant (Lebeis, 2014; Tikhonovich and Provorov, 2011). Plant rhizosphere is in constant connection with a variety of microbes (Vandenkoornhuyse et al., 2007), and several members cannot be cultivated under lab conditions. These microorganisms coevolved with their associated host plants and engineered their community structure accordingly (Lau and Lennon, 2012). It is therefore, presumed that the ecological interaction between the plant, soil and microorganisms are of paramount importance in assisting the growth and protecting against unfavorable circumstances for both partners (Qin et al., 2016).

The advantages of plant microbiome in agriculture, demands comprehensive studies on the interactions between plants, environment and microorganisms, agricultural practices. Further, usage of plant beneficial microbiomes for crop productivity (Schlaeppi and Bulgarelli, 2014; Berg et al., 2014), needs substantial improvements in experimental methods, characterization methods and modeling systems (Lebeis, 2015; Widder et al., 2016). However, still the molecular behavior and physiological aspects of the majority of microorganism are still uncharacterized. Efforts are also required to develop empirical and theoretical bases of forecasting the microbial community role keeping in view point the structural and functional responses to environmental deviation. Modern high-throughput tools permit fast and accurate identification of the functional attributes at multiple levels and operational taxonomic unit level to limited microbial community level, throughout the environmental deviations, are vital for filling the knowledge gap. Modeling and bioinformatics tools coupled to cutting edge omics technologies are vital importance for in-depth and accurate understanding and thereafter, effective utilization of these interactions (Fondi and Liò, 2015; Franzosa et al., 2015) for agricultural applications for countering tolerance to biotic and abiotic stresses (Singh et al., 2016; Wang et al., 2016). Modeling systems are critical for explaining the adaptive responses between plant and microbes which initiate the rhizospheric colonization or entophytic association. In addition, extensive studies are also required to describe the role of plant root exudates in structuring the microbial community and their functional behavior (Rosier et al., 2016). Already the studies on pathogenic and beneficial, have resulted major advances in plant immune responses still these experiments are mostly limited to characterization of one-on-one interaction. Further, experimental studies on microbial communities in the background of the structural and functional point will assist us in developing potential mechanisms for crop productivity based principles of ecology (Christian et al., 2015; Busby et al., 2017).

1.7 Functional aspects of plant beneficial microorganisms

The colonization of monocot and dicot crops by PGPR, help the host plants to tolerate abiotic and biotic stresses through diverse mechanisms (Ngumbi and Kloepper, 2016). The plant beneficial the microbiome effects plant health and productivity under abiotic stresses such as water stress and saline conditions. Microorganisms such some fungal members can even prolong their hyphae during mycorrhizal association one step ahead of plant roots, and then facilitate nutrients or water acquisitions (Ngumbi and Kloepper, 2016; Qin et al., 2016; Vurukonda et al., 2016). The other factors such as phytohormones production such as IAA, abscisic acid (ABA) and GA, and ACC-deaminase production to decrease the ethylene level in the roots also contribute benefits the host plant. *Azospirillum* spp., *Bacillus thuringiensis* and other microorganisms produce IAA themselves and hence can increase the lateral roots and root hairs formation, and hence help the plant to persist under water deficit conditions (Armada et al., 2014). Similar to this, ABA and GA-production by *Azospirillum lipoferum* can help maize plants to manage water loss by regulation of stomatal closures and stress signaling pathways (Cohen et al., 2009). The ACC-deaminase production by *Achromobacter piechaudii*

ARV8, is helpful in decreasing the stress levels in the tomato and pepper plants by metabolizing the 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene, into NH_3 and α -ketobutyrate (Shaharoona et al., 2006) helped the plants against drought tolerance (Mayak et al., 2004). The secretion of exopolysaccharides (EPS) layer increases plant productivity under water limited conditions (Lakshmanan et al., 2017).

1.8 Plant beneficial microbiome in soil

The continuous growth of crops in soils often leads to the accumulation of pathogens (Santhanam et al., 2015) or disease suppressive soils containing biocontrol agents as native bacterial community (Raaijmakers and Mazzola, 2016). The plant soil responses are found to rely on the type of fungi involved in mycorrhizal association or nitrogen fixing bacteria associated with plants (Bennett et al., 2017; Teste et al., 2017; Finkel et al., 2017). As it well established fact that plants perceive microorganisms via pattern-recognition receptors which interact with microbe-associated molecular patterns (MAMPs), and then activate the defense response to halt the pathogens and its activities (Jones and Dangl, 2006; Bohm et al., 2014). Therefore, majority non-pathogenic microorganisms associated to plants also possess their individual MAMPs, which can stimulate the plant basal immune response. However, it is unclear how plants manage to circumvent the beneficial microbiome via their immune response! The differences in transcripts profiling of *Arabidopsis* has been found upon inoculation with various non-pathogens of its natural microbiome (Vogel et al., 2016; Finkel et al., 2017). In another study, it was found that challenging tomato plants with *Ralstonia solanacearum*, led to change in root exudate profiling and enhanced production of phenolic compounds. Change in exudates profiling of plants are found associated with changes in soil microbial richness, and the reaction could be rivaled in vitro using phenolic compound such as caffeic acid (Gu et al., 2016). Similarly, inoculation of *Pseudomonas fluorescens* WCS417 in *Arabidopsis* planta led to variation in plant volatiles compounds against herbivorous caterpillar and attracted insecticidal wasps (Pangesti et al., 2015). Therefore, it can be assumed that plants are capable of discriminating pathogen and non-pathogen either by counterattacking microbial growth or by ignoring, or positively supporting it (Finkel et al., 2017).

1.9 Plant beneficial microbiome in agricultural crops

The microbial bioformulations have merged as an effective strategy than classical plant breeding and even modern genetic methods (Smith, 2014). In return, the plants by releasing carbon sources such as sugars in the form rhizodeposition, attracts the microbial communities for promoting their activities (Jones and Nguyen, 2009; Mendes et al., 2013). The details of plant beneficial attributes are beyond the scope of this book chapter and details of different beneficial microorganisms can be studied from some of the excellent reviews (Harman et al., 2004; Rosenblueth and Martínez-Romero, 2006; Lugtenberg and Kamilova, 2009; Rodriguez et al., 2009; Friesen et al., 2011; Singh et al., 2011; Bulgarelli, 2013; Qin et al., 2016; Sharma et al., 2017a,b).

1.10 Microbes mediated plant salt tolerance

Salinity negatively affects the plant growth. It is one of the limiting factor in increasing the crop yield. The application of microorganisms has gained attention, due to limitation of classical breeding programs for salt-tolerant cultivars (Qin et al., 2016). Plants, upon saline and abiotic stresses produce high amount of ethylene which drastically restrict the root growth (Mahajan and Tuteja, 2005). Although, molecular mechanisms of PGPR, responsible for enhanced salt tolerance in plants are not fully explored but the alteration in endogenous plant growth regulators is considered as key point (Kang et al., 2014). Studies also unravel that IAA- overproduction by *Sinorhizobium meliloti* strain, enhanced the *Medicago truncatula* plants growth in saline conditions (Bianco and Defez, 2009).

In comparison to ethylene, improved salt tolerance through IAA production is found to root proliferation (Dodd and Pérez-Alfocea, 2012). On the other side, in saline soil inoculation of PGPR, decreased ABA level in roots and also considerably changed the long-distance ABA flows from shoot-to-root phloem as well as root-to-shoot xylem (Dodd and Pérez-Alfocea, 2012; Jiang et al., 2012; Belimov et al., 2014), which might alleviate plant acclimation to drought. Inoculation of *Rhodococcus* sp. and *Novosphingobium* sp. metabolized ABA level in vitro (Belimov et al., 2014). Similarly, *Bacillus megaterium* strain enhanced the tomato plants growth in ABA- mutants (Porcel et al., 2014). Therefore, experimental studies revealed that PGPR producing growth regulators, PGPR metabolizing growth regulators and other PGPR acts in a different way for modulating plant growth regulators level and therefore can cause different plant reactions to salinity (Fig. 1.3). Increase in nutrients under saline conditions, inoculation of *Achromobacter piechaudii*, led to enhanced N&P (Mayak et al., 2004) whereas *Bacillus aquimaris*, led to increase in N, P & K in wheat

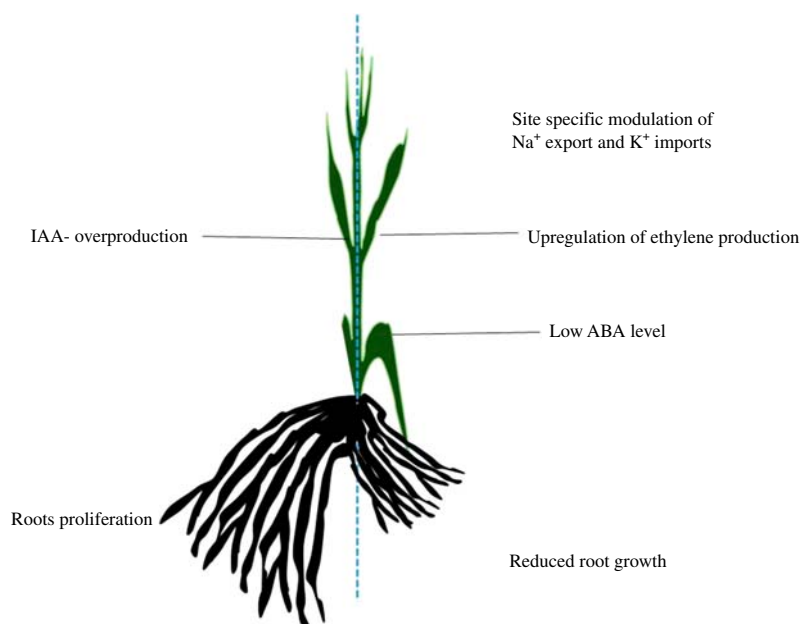


FIGURE 1.2 Role of plant beneficial microorganisms producing growth regulators, PGPR metabolizing growth regulators, and other PGPR acts in a different way for modulating plant growth regulators level and therefore causes different plant reactions to salinity. PGPR enhance the Na⁺ export and K⁺ imports exopolysaccharides through the biofilm formation on plant root surface which restrict the Na⁺ imports into roots.

leaves under saline stress (Upadhyay and Singh, 2015). Siderophore production also helps plant in scavenging Fe³⁺ which can be easily transferred to the host plant (Kloepper et al., 1980) (Fig. 1.2).

Regulation of Na⁺ level is vital to maintain regular plant growth in saline stress. A number of PGPR are found to enhance the Na⁺ export and K⁺ imports in plants (Rojas-Tapias et al., 2012; Han et al., 2014). The exopolysaccharides secreted by PGPR, facilitate the biofilm formation on plant root surface, restrict the Na⁺ imports into roots (Ashraf and Harris, 2004; Dodd and Pérez-Alfocea, 2012). The inoculation of *B. subtilis* strain GB03 in Arabidopsis plants, caused downregulation of K⁺ transporter i.e. HKT1 in the plant roots (Zhang et al., 2008). Contrary to this, GB03 strain, facilitated shoot-to-root Na⁺ equilibrium by the induction of HKT1 in shoots (Zhang et al., 2008). The dual site specific modulation of HKT1 in plants, by GB03 strain, revealed the role of plant beneficial microorganisms in Na⁺ homeostasis (Zhang et al., 2008) (Fig. 1.2). The transcriptomic and microscopic studies also revealed the behavior of *Stenotrophomonas rhizophila* DSM14405T in salt stress (Gao et al., 2012) whereas a combination of N₂-fixing bacteria and AM fungi, facilitate the legume plants in countering abiotic stresses such as drought (Aliasgharzad et al., 2006). The beneficial effects of inoculation of *A. brasilense* with AM fungi has been observed in tomato, maize and cassava (German et al., 2000; Casanovas et al., 2002; Creus et al., 2005; Bhardwaj et al., 2014).

1.11 Holistic view of plant, microbiome and environment interactions

The description of plant beneficial microbiome, depend on the ecological challenges. Microbiome with universal plant growth promoting potential improves the plant growth and their ability to counter different stresses. The challenges to engineer plant beneficial microbiome require to be examined. The biotic and abiotic parameters of soils can be modified by the usage of soil additives, land preparation for agriculture, and cropping system. The three ways interactions between microorganisms and the environment followed by management are vital factors during microbiome use for improving plant vigor and yield. The challenges of beneficial microbiomes implementations for agriculture are governing by host genotype-and-environment interaction. The effect of host plant genotype on the microbiome can differ, across a wide range of environmental surroundings, and even across plant tissues (Peiffer et al., 2013; Wagner et al., 2016). Another key challenge associated to the application of microbiome environmental variations in agricultural fields which demands a resilient system. An in-depth understanding of microbiome can help in portraying the underlying cause that why and how the microbial consortium is well adapted in one climatic condition but does not perform well in another environment which may be due to a single parameter such as pH of soil (Fierer and Jackson, 2006). Moreover, the behavior to its surrounding community, across farm lands, management practices adopted (Soman et al., 2016) and climate change (Barnard et al., 2013; DeAngelis et al., 2015) also help in determine the resilience of beneficial microbiome. Therefore, an principal point is to describe microbial consortia which can perform under diverse ecosystems (Busby et al., 2017). A competitive PGPR needs to conquer and perform against the local microbial population

and environmental conditions, for developing compatible and successful interaction. Understanding of plant microbiome successional dynamics of how during the growing season, this soil-microbial community undergoes succession changes at above-ground (Shade et al., 2013; Copeland et al., 2015) and below-ground (Edwards et al., 2015) needs to be explored. Estimation of the persistence of microbial inoculants needs to be established within a complex community (Finkel et al., 2017).

1.12 Public interest and the future of plant beneficial microbiome

In almost all of the developing nations including Asia, Latin America and Africa, legislation or standards on the implementation are not followed and plant beneficial microbes are generally prepared using non-sterile carriers. In other countries such as Argentina, Rwanda, Brazil where standards and guidelines are present, they are either not implemented in producer's techniques (Bashan, 1998; Hungria et al., 2005). In order to translate their potential in fields, the inoculum must hold its anticipated abilities (Xavier et al., 2004). One of major reason involves the rapid decrease in the viable cell count and often reaches below threshold and formulation becomes unproductive in agricultural fields. A promising inoculum consist of more than 10^3 colony count per gram of soil (Ben Rebah et al., 2002). To achieve such high colony count, it is necessary to use carriers with features such as good aeration, have better water-holding capacity, and can maintain microbial growth as well as self-life. Moreover, the carrier must be cheap, easy to use and accessible (Bashan, 1998; Ben Rebah et al., 2002). The liquid formulations, consist of specific broth 10–40%, of dispersant 1–5%, ingredient for suspension 1–3%, surfactant at 3–8% and carrier liquid either oil or water a combination of both at 35–65% of by weight. For achieving viscosity to avoid setting of contents colloidal clays, starch cellulose or synthetic polymers, polysaccharide gums are recommended (Kalra et al., 2010) (Fig. 1.3). For plant probiotics, it is also mandatory that the microorganisms, exhibit positive roles in plant growth but at the same time it should be harmless for humans and our ecosystem. A polyphasic method including phenotypic, chemotaxonomic and genotypic categorization must be used to record for the microorganisms for plant probiotics (Herrmann et al., 2013). For strain characterization, use of molecular methods including 16 S rDNA or internal transcribes spacer (ITS) sequencing DNADNA hybridization should be used (Young et al., 2012). It should be mandatory to follow the policies of the safety assessment charter set by the American Biological Safety Association (ABSA). Prior to their release, filed demonstrations and methodical instruction to farmers can help in building trust and proper usage of inoculants to them. It will provide vision built on the agricultural fields of the farmers, and marketing of products for efficient implementation of technology (Sanap et al., 2009). ultimately can lead to considerable growth in the use of bioformulations for agricultural fields (Kannaiyan, 2003; Bhattacharyya and Jha, 2012; Bharti et al., 2017).

Plant beneficial microorganisms, due to lack regulatory frameworks/guidelines are difficult to evaluate for quality and efficiency. Therefore, often low-quality and inefficient bioformulations have been found (Herrmann et al., 2013).

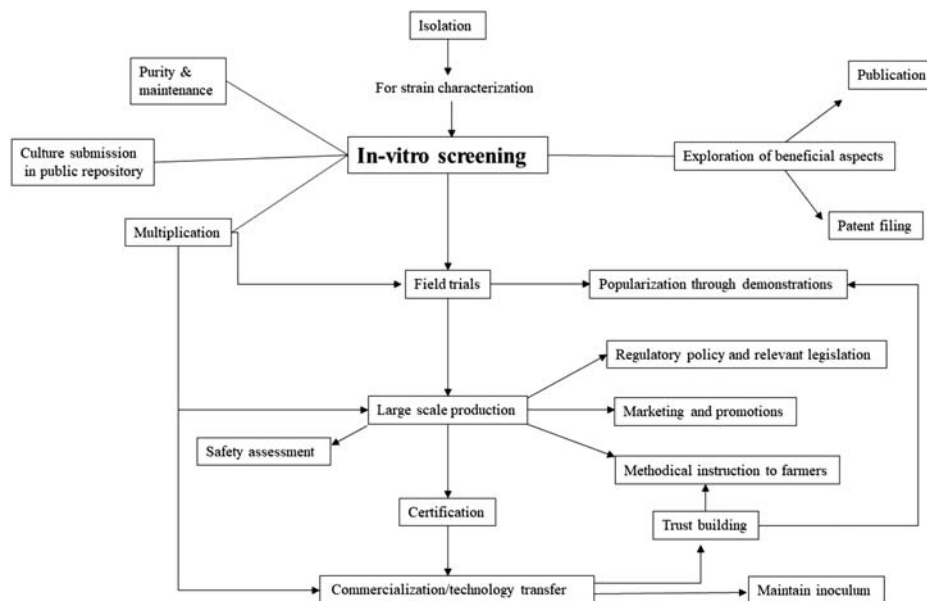


FIGURE 1.3 Outline of exploration of plant beneficial microorganisms.

Due to lack of universal processes for beneficial microbial implementations, the prevailing policies and recommendations are largely restricted on rhizobial inoculants only that vary across the nations (Jenkins and Grzywacz, 2000; Lupwayi et al., 2000; Stephens and Rask, 2000). These policies merely represent the lowest cells count for inoculation per unit of inoculant weight and permit the maximum amount of impurities in final product (Lupwayi et al., 2000). In few nations, the policies for the commercialization of bioformulations include stringent regulatory procedures (Hungria et al., 2005) and a sovereign lab evaluates the microbial products for these criteria. The main obligatory recommendations for the applications of plant beneficial rhizobia include that first-all inoculants must ascertain that they are safe to the ecosystems. Secondly, preparation of inoculum should be from pure cultures and aseptic carriers, consists of at least 10^6 active cells per seed without any contaminants (Catroux and Amarger, 1992; Bashan, 1998).

However, Canada monitors a moderate quality regulatory policy and relevant legislation which imposes cataloguing of product and frequent random sampling and quality evaluation (Herridge, 2008). Minimum viable cell count in the range of 10^3 to 10^5 depending upon size of the seeds, however it lacks guidelines on product contamination (Olsen et al., 1994). In other nations including Australia, Thailand, and South Africa, the quality assessment programs are not obligatory. Australia, recommends up to 10^5 rhizobia per seed as obligatory and adequate with impurity level of 0.1% of the entire microbial population (Bashan, 1998). In Thailand, non-sterile carriers are usually used with cell count per seed 10^5 – 10^6 rhizobia per seed (Herridge, 2008). In USA and UK, there are no regulatory guidelines on the implementation and therefore, the quality of beneficial microbes is considerably variable (Date, 2000).

1.13 Conclusion

Plant productivity worldwide needs to be optimized, for high crop yield under constant changing climatic conditions. The synthetic fertilizers and pesticides need to be replaced with environmental friendly, efficient and sustainable and potential solutions (Farrar et al., 2014). The potential of plant beneficial microorganisms is abundant and well beyond the mass multiplication (Wiesman, 2009; Mazid and Khan, 2014). In an ecosystem, plants network in symbiotic association with cultivable and uncultivable diversity of microorganisms efficiently as phytobiomes. Collectively mentioned as the plant microbiome, these microorganisms are second genome to the plants and offers untapped prospective for plant growth and productivity (Berendsen et al., 2012). The microbiome includes an wide and functionally diverse gene pool, of prokaryotes, and eukaryotes, individually linked with distinct plant tissues or organs as endophyte or those residing in the soil immediately adjacent to the root surface, identified as the rhizosphere (Rout and Southworth, 2013) conceptualized as the soil area, either directly growing or responding to root exudates through a process called “rhizodeposition (Lakshmanan et al., 2017). The association of legumes crops, nitrogen fixing bacteria and mycorrhizal associated fungi is an example of tripartite symbioses in ecosystem. The fixation of nitrogen by rhizobia is energy extensive process and requires P for ATP generation which is obtained from fungal partner” (Hunter, 2016). The beginning of culture-independent techniques in microbial ecology, coupled to high-throughput data generation and analytic tools, enabled us to carry out systematic understanding of plant microbiome and how it can be explored. Although mostly a black box, the microbial diversity and its assembly and the species richness in different plants and soil compartments has been explored in the Arabidopsis (Bulgarelli et al., 2012a,b; Lundberg et al., 2012; Bai et al., 2015; Lebeis et al., 2015), and certain cultivated crops (Peiffer et al., 2013; Bulgarelli et al., 2015; Edwards et al., 2015; Lakshmanan et al., 2017). The unification of dynamic community of plant microbiome and its evolution to ecological instabilities continuously challenge the efficient functioning of phytobiome (Lakshmanan et al., 2017).

Although, several studies have been conducted to the role of soil microbiome in improving the plant health and productivity against biotic and abiotic stresses (Shanmugam et al., 2008; Sharma and Salwan, 2015; Salwan et al., 2019). The Genomic Standards Consortium (Field et al., 2011), American Academy of Microbiology (Reid and Greene, 2013), Unified Microbiome Initiative (Alivisatos et al., 2015), Report on the Fast-Track Action Committee on Mapping the Microbiome (Stulberg et al., 2016), American Phytopathological Society 2016, Earth Microbiome Project (Gilbert et al., 2014), and National Microbiome Initiative (Handelsman, 2016) have each called for the standardization of data collection, processing, and analysis. The efforts are underway with direction from the U.S. National Institute of Standards and Technology. Several of these initiatives also defined knowledge gaps and research priorities in plant microbiome studies. The initial efforts were on the discovery and depiction of taxonomic diversity, whereas in recent times emphasis has been given on the functional aspects of microbiomes (Busby et al., 2017).

Nevertheless, efforts are still required to fill the gaps on several aspects of plant-and their interaction with microbiome. For example, i-the development of host–microbiome model systems for both for agricultural crops and non-agricultural crops, ii- their association to microbiome and reference genomes, defining cultivatable core microbiomes as well as no cultivable metagenomes in these model systems, iii-characterization of functional behavior of microbiome in

plant-microbiome interactions, iv-characterizing the tripartite interaction amid plant genotype-microbiome and environment needs investigation. These extraordinary complexities of plant microbiome imposes a daunting task to completely understand the functionality of plant-associated microbiomes (Busby et al., 2017). In addition, developing rational strategy can help us to improve the resilience of plant beneficial microorganisms in extreme environmental conditions (Lakshmanan et al., 2017).

Beside genetic potential of the microbiomes, the process of microbial inoculation may play role by improving delivery of strain through seed coating, clay particle, and peat based methods. The efforts to maximize colonization should also guarantee against negative affect on different components of ecosystem (Busby et al., 2017). Even though agriculture specialists support the profits of plant beneficial microorganisms for plant growth and productivity, the distribution and acceptance of the technology to end users i.e. farmers, failed to achieve its actual potential (Dennis et al., 2010). Therefore, the farmers' awareness, trust and outlook towards natural solution which are often not taken into the consideration are of supreme importance to the practice of microbiome. As revealed by Keith Andrews and his colleagues that exploration of plant beneficial microorganisms has failed largely, due to lack of communication with farmers (Andrews et al., 1992). The effective experiments based approaches involving farmers, importance on social science investigation and marketing beneficial microorganisms through information and communication technologies (ICT), will help in the popularization of these microbes. Apprehensions about sustainable agricultural ecosystems, demands to develop technologies and practices without adverse effects on our surrounding environmental, and are easily accessible to farmers. Despite the major progress in agricultural productivity in the past 50 years, it would be over-optimistic to pre-assume it will work in a linear fashion over the period of time. The plant microbiome manipulation is largely limited to certain microorganisms. Unfortunately, the introduction of such microbes either single strain to soil is often found unproductive in plant growth promotion and alleviating stress tolerance, mainly because of competition with native soil microbial communities and restricted colonization efficiency.

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Chapter 2

Plant beneficial microbes: do they have a role as antiviral agents in agriculture?

Despoina Beris and Nikon Vassilakos

Laboratory of Virology, Department of Phytopathology, Benaki Phytopathological Institute, S. Delta 8, 14561 Kifissia, Athens, Greece

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2.1 Introduction

Viruses are among the most important plant pathogens with half of the emerging epidemics having viral etiology and resulting in great economic losses about 60 billion US dollars per year worldwide (Song et al., 2010). Furthermore, trade globalization and climate change have led to spread of virus diseases to new areas, or to the emergence of virus variants with expanded host-range (host-jump events). To date, therapeutical measures against plant-virus diseases in the field are not available, therefore, the main control strategy used in practice is based on prevention. Genetic resistance is by far the most effective way to control plant viruses. However, ‘traditional’ genetic sources of resistance to viruses are rare (Lecoq et al., 2004) and due to the high rate of mutation of the viral genomes this resistance even when applicable, is frequently broken under field conditions. The control of virus insect vectors constitutes the second important mean of virus disease management, however to be effective requires considerable economic resources while it has a huge environmental impact. Thus, the identification of novel durable and eco-friendly approaches for the management of virus diseases in the field is imperative for a sustainable agriculture.

Plant viruses are obligate, intercellular pathogens that rely on host factors to complete their life cycle while they manipulate the host physiology in every step of their infection cycle [i.e. replication, encapsidation, cell-to-cell movement, systemic accumulation, vector transmission (Caranta et al., 2011)]. The outcome of a viral infection as well as the intensity of the symptomatology, depend on a complex network of specific interactions between the viral and the host proteins. During a co-evolutionary race, plants have evolved a sophisticated network of defense mechanisms to battle viral infection and restrict viral replication and movement [i.e. RNA silencing, effector-triggered immunity (ETI), innate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), reviewed in Calil and Fontes (2017)].

2.2 The role of plant hormones in defense against viruses

The precise regulation of plant response pathways induced during virus infection is performed by the main plant hormones, namely, salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET). Hormones act as

signaling molecules that stimulate downstream response pathways resulting in massive transcriptional reprogramming. As counter defense measures, viruses disrupt plant hormone regulation (Alazem and Lin, 2015). Salicylic acid (SA) is a key-factor of the ETI response and RNAi defense. During ETI, the recognition of the viral effector by the *R* protein activates the SA biosynthesis and signaling. SA mediates the induction of several pathways such as accumulation of reactive oxygen species (ROS), secondary wall modifications and callose deposition, programmed cell death, hypersensitive reaction and accumulation of pathogenesis-related proteins (PR) (Alazem and Lin, 2015). In addition, SA is the signaling molecule of systemic acquired resistance (SAR) that protects the uninfected plant parts from upcoming viral attacks. In many plant/virus pathosystems, the disruption of the SA biosynthesis and signaling pathways results in susceptibility of the host even in the presence of an *R* gene. For example, in potato the *Ny1* resistance gene conferring resistance against *Potato virus Y* (PVY), results in a hypersensitive reaction with the restriction of the virus in necrotic lesions. On contrary, transgenic *Ny1* potatoes expressing the salicylate hydroxylase (*NahG*) are susceptible to PVY, due to disruption in the SA accumulation. This demonstrates that even in the presence of *Ny1* gene, SA plays an indispensable role in the *Ny1*-mediated resistance (Baebler et al., 2014).

Jasmonic acid (JA) shows diverse function in virus infection that depends on the infection stage, the host species and the virus genus. In PVY/*Potato virus X* (PVX) double infections the exogenous application of JA at the early stages of infection led to enhanced resistance but with no difference made at the latter stages or in symptom development (García-Marcos et al., 2013). In *Arabidopsis thaliana*, the continuous application of JA in all infection phases resulted in a lower titer of *Tomato yellow leaf curl Sardinia virus* (TYLCSV). In contrast, in the tobacco/*Tobacco mosaic virus* (TMV) pathosystem, the disruption of the JA signaling pathway resulted in elevated resistance and application of JA led to the impairment of the *N*-mediated resistance against the virus (Oka et al., 2013).

Absciscic acid's role on virus infection is also diverse. At the early phases ABA can enhance the defense status of plants against several pathogens through callose deposition and stomatal closure. However, at latter stages, its antagonistic role against JA and SA results in reduced ROS accumulation and negative effects in plant defense (Alazem and Lin, 2015). Although the role of ABA has been extensively studied in other pathogens, its role in plant-virus interaction is not well characterized. In many cases, the induction of ABA was suggested to confer an elevated defense status and to be involved in HR reactions. ABA increased biosynthesis after chitosan application in common bean resulting in an enhanced defense status during the early stages of *Tobacco necrosis virus* (TNV) infection (Iriti and Faoro, 2008). Moreover, several studies have reported that ABA is involved in the reduction of viral spread through the strengthening of plasmodesmata, a plant cellular structure involved in the viral cell-to-cell movement (Alazem et al., 2017; Mauch-Mani and Mauch, 2005). Recent studies have also demonstrated that the antiviral RNAi machinery is partially regulated by ABA through the induction or reduction of the AGO transcriptional levels (Alazem et al., 2017; Li et al., 2012).

The role of ethylene (ET) in plant/virus interaction is the least investigated. In general, ET is proposed to be linked or have similar effects with JA. In the case of *Arabidopsis*/*Cauliflower mosaic virus* (CaMV) pathosystem. ET is responsible for the development of disease symptoms (Geri et al., 2004). In the same host, impairment of the ET pathway results in resistance against TMV, due to restriction of its systemic movement (Chen et al., 2013) and on contrary, in tobacco, ET promotes the formation of local lesions and resistance against the same virus (Ohtsubo et al., 1999). Thus, the role of ET during virus infection depends on the pathosystem, as the same virus may be differentially related with ET signaling in distinct hosts or the same host may regulate differentially the expression of ET associated genes upon infection with viruses belonging to distinct genera.

2.3 Plant growth promoting microbes (PGPM)

Plant growth promoting microbes (PGPM) consisting of rhizobacteria (PGPR) and fungi (PGPF) have gained an increasing importance in agriculture, due to their beneficial effect on plant physiology. PGPMs can directly promote plant growth through the production of nutrients that are usually scarce in the rhizosphere. Application of PGPM as bio-fertilizers results increased nitrogen fixation, solubilization of phosphorus and uptake of potassium, which are the key macronutrients for plant development. In combination with the production of nutrients, PGPM can modulate or produce plant hormones such as auxin, cytokinin, gibberellin, ethylene and absciscic acid. These hormones are the key regulators of plant development and their perception in the root system results in modifications of root architecture to increase nutrient uptake that finally promote plant growth.

PGPM's indirect mode of action is based on their ability to confer tolerance against abiotic stresses. The interaction of plants with PGPM results in alterations in plant secondary metabolism and increased production of antifreeze proteins, antioxidant enzymes and proline and polyamine biosynthesis. These metabolic compounds are linked to the tolerance of plants in several abiotic stresses, such as freeze, heat and drought. Importantly, PGPM can also induce plant

resistance against different sources of biotic stress, and they have been already used commercially in agriculture against several fungal and bacterial diseases. Briefly, their mode of action is based on (i) the production of siderophores, bacteriocins, antibiotics and hydrolytic enzymes, (ii) antagonism with soil-borne pathogens, (iii) the production of plant hormones and phytoalexins, (iv) the promotion of plant growth and finally (v) the induction of systemic resistance (ISR) (Meena, 2012). ISR refers to an enhanced defense status which is characterized by the activation of defense associated genes and compounds of the phenylpropanoid pathway (Meena, 2012), during which plants are better prepared to tackle upcoming pathogen attacks. Notably, despite an accumulating number of studies reporting different levels of antiviral activity of PGPM, thus far PGPM have not been used commercially against viruses. This chapter aims to present and highlight the potential of PGPMs as antiviral agents. A description according to virus species or genus tested, follows. The multifaceted antiviral mode of action of PGPMs as revealed thus far is illustrated in Fig. 2.1, whereas important aspects of all cited publications are presented in Table 2.1.

2.4 Studies of PGPMs against viruses

2.4.1 Cucumber mosaic virus

Most studies of antiviral action of PGPMs concern *Cucumber mosaic virus* (CMV). CMV is the type member of genus *Cucumovirus* (family *Bromoviridae*). In nature, it is transmitted by more than 80 species of aphids in a non-persistent manner and to a lesser extent by seed. CMV can infect more than 1200 plant species belonging to 100 families causing severe diseases in many agronomically important crops. In *Arabidopsis* plants, Ryu and co-workers (Ryu et al., 2004) showed that application of *Serratia marcescens* strain 90-166 and *Bacillus pumilus* strain SE34, under greenhouse conditions, through drenching of soilless potting medium, resulted in milder CMV symptomatology and reduced systemic accumulation of the virus. The molecular mechanism of the resistance induced by strain 90-166 was investigated using SA-deficient and JA-insensitive *arabidopsis* mutants. Application of the bacterial strain in the JA-insensitive mutant resulted in increased susceptibility to CMV in a similar manner to the untreated wild-type plants, whereas the same application in the SA-mutants resulted in reduced CMV accumulation. The observed differential response of these mutants indicated that JA-dependent pathway was induced. Furthermore, the subsequent gene expression analysis of the two main gene markers of SA and JA pathways, *Pathogenesis-related protein 1* (*AtPR1*) and *Plant defensin*

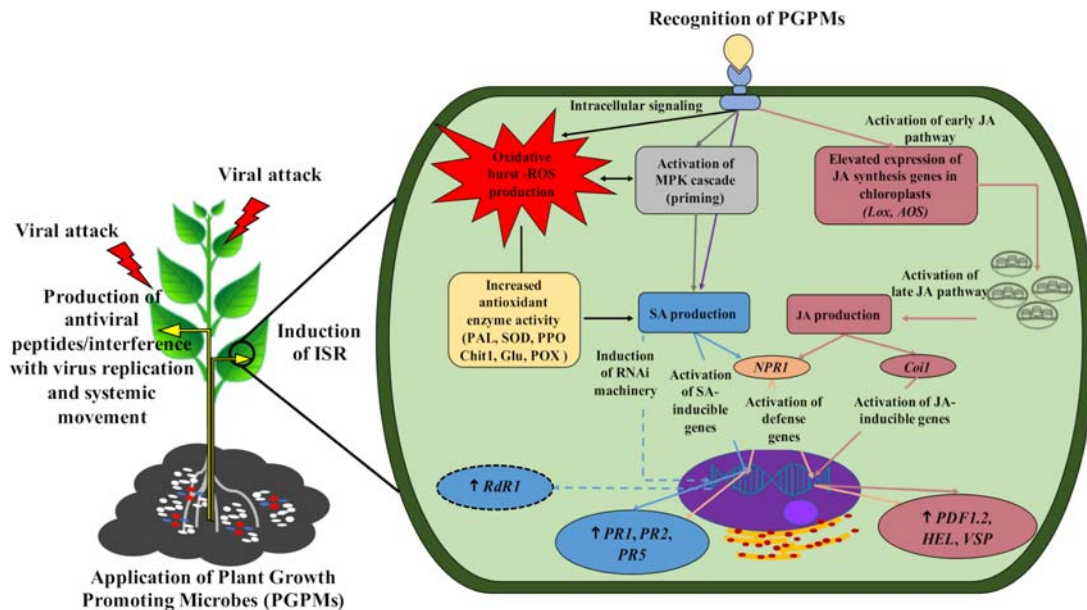


FIGURE 2.1 Schematic representation of the mode of action of plant growth promoting microbes (PGPMs) against viruses. The direct effect of PGPM application against viruses is based on the production of antiviral peptides, and in some cases of endophytic PGPM interfere with virus replication and movement. Most importantly, PGPM induce systemic resistance (ISR). On the intracellular level, recognition of PGPM-produced elicitors by plant receptors triggers several defense pathways based on oxidative burst and ROS production, priming and mainly to the elevated production of the SA and JA hormones. The latter results in the enhanced expression of the main defense genes such as pathogenesis-related proteins (PRs) and plant defensins (PDF1.2).

TABLE 2.1 Synopsis of the studies described in the text, which involved experiments with plant growth promoting microbes (PGPM) and plant viruses; n/t, not tested.

Genus	Virus	Host	PGPM	Type of resistance	Molecular mechanism	Experimental conditions	Reference
<i>Cucumovirus</i>	<i>Cucumber mosaic virus (CMV)</i>	Cucumber	<i>P. fluorescens</i> 89B-27, <i>S. marcescens</i> 90-166	Reduced disease incidence	n/t	Greenhouse	(Raupach, 1996)
			Mixtures of <i>B. amyloliquefaciens</i> , <i>B. sphaericus</i> & <i>B. pumilus</i> / individual application of <i>B. amyloliquefaciens</i> IN937a & IN937b	Delay of symptom development & reduced symptom severity	n/t	Greenhouse/Field	(Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003)
			<i>B. subtilis</i> , <i>P. fluorescens</i> & <i>A. chroococcum</i>	Reduced disease incidence & lower virus titer	Increased peroxidase & β -1,3-glucanase enzyme activities	Greenhouse	(El-Borollosy and Oraby, 2012)
			<i>F. equiseti</i> GF18-3 & <i>G. mosseae</i>	Reduced virus accumulation	Induction of SA pathway Induction of JA pathway only in presence of <i>G. mosseae</i>	Greenhouse	(Elsharkawy et al., 2012a)
		Tomato	<i>P. fluorescens</i> 89B-27, <i>S. marcescens</i> 90-166	Reduced disease severity	n/t	Greenhouse	(Raupach, 1996)
			Formulation of <i>B. pumilus</i> T4 & INR7, <i>B. amyloliquefaciens</i> IN937a & <i>B. subtilis</i> IN937b supplemented with chitosan	Reduced virus accumulation, milder symptoms	Growth promotion	Greenhouse	(Murphy et al., 2003)
			<i>B. pumilus</i> INR7, <i>B. amyloliquefaciens</i> IN937a, <i>B. subtilis</i> IN937b, <i>K. cryocrescens</i> IN114	Delayed disease progress	n/t	Field	(Zehnder et al., 2000)
			<i>B. subtilis</i> OKBHF	Reduced disease severity	Induction of expansin genes & defense-related genes <i>Pti4</i> & <i>Pti6</i>	Greenhouse	(Wang et al., 2011)
		Tobacco	<i>P. chlororaphis</i> O6	Lower virus titer	n/t	Greenhouse	(Ryu et al., 2007)
			<i>P. lentimorbus</i> B-30488	Reduced virulence & virus systemic accumulation	Restoration of genes up-regulated by CMV and lower ROS production	Greenhouse	(Kumar et al., 2016)
			<i>P. simplicissimum</i> GP17-2	Reduced disease severity & virus accumulation	Induction of SA & JA/ET pathways	Growth chamber	(Elsharkawy et al., 2012b)
		Arabidopsis	<i>P. simplicissimum</i> GP17-2	Reduced disease severity & virus accumulation	Induction of SA & JA/ET pathways	Growth chamber	(Elsharkawy et al., 2012b)
			<i>S. marcescens</i> 90-166 & <i>B. pumilus</i> SE34	Reduced symptom severity & virus accumulation	Induction of JA pathway	Greenhouse	(Ryu et al., 2004)

Potyvirus	Potato virus Y (PVY)	Potato	<i>B. vallismortis</i> EXTN-1	Reduced incidence & disease severity	Induction of SA- and JA- defense pathways	Field	(Park et al., 2006)
	Bean yellow mosaic virus (BYMV)	Faba bean	<i>P. fluorescens</i> FB11 & <i>R. leguminosarum</i> bv. <i>viceae</i> FBG05	Reduced incidence & virus concentration in challenged leaves	Increased production of SA and peroxidase enzyme activity	Greenhouse	(Elbadry et al., 2006)
	Bean common mosaic virus (BCMV)	Cowpea	<i>B. pumilus</i> T4 & <i>B. subtilis</i> GBO3	Reduced incidence	n/t	Screenhouse/ Field	(Udaya Shankar et al., 2009)
	Zucchini yellow mosaic virus (ZYMV)	Squash	<i>B. bassiana</i> ATCC 74040, ATP01, ATP05 & EABb04/01-T	Reduced disease severity & delayed symptom development	n/t	Growth chamber	(Jaber and Salem, 2014)
	Watermelon mosaic virus (WMV)	Pumkin	<i>B. subtilis</i> 281 & <i>B. pumilus</i> 293	Reduced disease severity, symptom development & virus concentration	n/t	Greenhouse	(Elbeshehy et al., 2015)
	Papaya ringspot virus (PRSV)	Squash	<i>B. amyloliquefaciens</i> IN937a, <i>B. subtilis</i> 1216c & IPN-19, <i>B. pumilus</i> SE34 & T4, <i>B. sphaericus</i> SE56 (individually or in combination)	Reduced disease severity	n/t	Greenhouse	(Abdalla et al., 2017)
	Turnip mosaic virus (TuMV)	Leaf mustard	<i>P. fluorescens</i> & <i>B. subtilis</i>	Delay in symptom development & reduced disease severity	Increased catalase activity	Greenhouse	(Diyansah et al., 2017)
Tobamovirus	Tobacco mosaic virus (TMV)	Hot pepper	<i>B. cereus</i> , <i>Bacillus</i> sp. & <i>B. sanguinis</i>	Delay in symptom development, reduced disease severity & virus concentration	Increased peroxidase activity	Greenhouse	(Damayanti et al., 2007)
		Tobacco	<i>B. subtilis</i> G1	Delay in symptom development, reduced disease severity & virus concentration	Induction of both SA- & JA- defense pathways	Greenhouse	(Wang, 2009)
	Pepper mild mottle virus (PMMoV)	Tobacco	<i>B. amyloliquefaciens</i> EXTN-1	Increased number of asymptomatic plants	Induction of a mechanism similar to the N-mediated resistance. Induction of <i>NtPR1a</i> , <i>NtPAL</i> and <i>NtHMGR</i>	Greenhouse	(Ahn et al.)
	Cucumber green mottle mosaic virus (CGMMV)	Cucumber	<i>S. maltophilia</i> HW2	Reduced disease index & symptom severity, significant delay in virus replication	Increased levels of <i>SOD</i> , <i>CAT</i> , <i>PR1</i> & <i>PR5</i>	Greenhouse	(Li et al., 2016a)

(Continued)

TABLE 2.1 (Continued)

Genus	Virus	Host	PGPM	Type of resistance	Molecular mechanism	Experimental conditions	Reference
<i>Begomovirus</i>	<i>Tomato yellow leaf curl virus</i> (TYLCV)	Tomato	<i>E. asburiae</i> BQ9	Reduced virus load	Rapid and robust oxidative burst accompanied with elevated levels of PAL enzyme activity & <i>PR1a</i> & <i>PR1b</i> transcripts.	Greenhouse	(Li et al., 2016b)
			<i>B. amyloliquefaciens</i> Ba13	Reduced infection rate, delay in symptom development, reduced virus load	Increase POD, PPO, & PAL enzyme activities. Continuous & rapid induction of <i>PR1</i> , <i>PR2</i> & <i>PR3</i>	Greenhouse	(Guo et al., 2019)
<i>Tospovirus</i>	<i>Tomato mottle virus</i> (ToMoV)	Tomato	<i>B. pumilus</i> SE34, <i>K. cryocrescens</i> IN114, <i>B. amyloliquefaciens</i> IN937a & <i>B. subtilis</i> IN937b	Reduced disease severity, symptom development & virus incidence	Reduction of whiteflies densities	Field	(Murphy et al., 2000; Zehnder et al., 2001)
	<i>Tomato spotted wilt virus</i> (TSWV)	Tomato	<i>P. fluorescens</i> COT-1, CHAO & COP-1 (individually applied or in mixtures)	Reduced virus incidence	Increased levels of PAL, PPO, glucanase & chitinase enzyme activities	Greenhouse/Field	(Kandan et al., 2002, 2005)
			<i>P. putida</i> C13	Reduced virus incidence	n/t	Field	(Zaccardelli et al., 2011)
	<i>Tomato chlorotic spot virus</i> (TCSV)	Tomato	<i>B. amyloliquefaciens</i> IN937a, <i>B. subtilis</i> 1216c & IPN-19, <i>B. pumilus</i> SE34 & T4, <i>B. sphaericus</i> SE56 (individually or in combination)	Reduced disease severity	n/t	Greenhouse	(Abdalla et al., 2017)
<i>Babuvirus</i>	<i>Banana bunchy top virus</i> (BBTV)	Banana	<i>P. fluorescens</i> CHAO	Reduced virus incidence	Increased accumulation of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, PR proteins, chitinase, β -1,3-glucanase and phenolics	Greenhouse/Field	(Kavino et al., 2008) (Harish et al., 2008, 2009)
			<i>Pseudomonas</i> sp. strain mixtures, EPB5 + EPB22 + Pf1 + CHAO, EPB5 + Pf1 + CHAO & EPB22 + Pf1 + CHAO	Reduced virus incidence			
<i>Potexvirus</i>	<i>Potato virus X</i> (PVX)	Potato	<i>B. vallismortis</i> EXTN-1	Reduced incidence & disease severity	Induction of SA- and JA- defense pathways	Field	(Park et al., 2006)
<i>Ilarvirus</i>	<i>Tobacco streak virus</i> (TSV)	Cowpea	<i>B. amyloliquefaciens</i> VB7	Reduced disease incidence	Growth promotion, ISR and secretion of antiviral peptides	Field	(Vinodkumar et al., 2018)

1.2 (*AtPDF1.2*) respectively, showed an elevated expression of *PDF1.2* in wild-type plants upon application of the 90-166 strain. Therefore, *S. marcescens* induced a JA-dependent and SA-independent defense pathway in Arabidopsis that resulted in the resistance against CMV (Ryu et al., 2004).

In studies with cucumber plants in 1996, the application of *Pseudomonas fluorescens* 89B-27 and *S. marcescens* strain 90-166 reduced CMV incidence under greenhouse conditions, whereas its application in tomato plants decreased virus accumulation (Raupach, 1996). Later, Jetiyanon and co-workers (Jetiyanon and Kloepper, 2002) demonstrated that the use of compatible mixtures of *Bacillus amyloliquefaciens*, *B. sphaericus* and *B. pumilus* strains as well as individual application of *B. amyloliquefaciens* strains IN937a and IN937b in cucumber seeds resulted in delay of symptom development in challenged leaves and more importantly in reduced symptom severity in the newly developed leaves under greenhouse conditions (Jetiyanon and Kloepper, 2002). In addition, application of the two *B. amyloliquefaciens* strains in cucumber plants provided a significant disease suppression in a two-year field experiment. Simultaneously, bacterization of cucumbers resulted in plants with increased biomass and fruit production (Jetiyanon et al., 2003). Application of *B. subtilis*, *P. fluorescens* and *Azotobacter chroococcum* strains through bacterization of cucumber seeds resulted, an increased number of uninfected plants after challenge inoculation with CMV. Moreover, in treated plants infected with CMV the titer of the virus was significantly lower compared to the untreated control plants and this resulted in reduced disease severity. Enzymatic analysis of uninfected *Azotobacter* and *Pseudomonas* treated plants showed increased peroxidase and β -1,3-glucanase enzyme activities that were linked to reinforcement of plant cell walls and induction of ISR (El-Borollosy and Oraby, 2012).

Until now two growth promoting fungi have been reported to induce systemic resistance in the cucumber/CMV pathosystem. Application of *Fusarium equiseti* GF18-3 and *Glomus mosseae* (Gm), in mixture or individually, resulted in reduced CMV accumulation, compared to untreated plants. Gene expression analysis of the defense associated genes, *PR-1a*, peroxidase (*POX*), lipoyxygenase (*LOX1*), phenylalanine ammonia lyase (*PAL1*), chitinase (*Chit1*) and β -1,3-glucanase (*Glu*) revealed an increase of the SA-associated *PR1-a*, *Chit 1*, *Glu* and *POX*. Interestingly, the induction of the JA-inducible genes *LOX1* and *PAL1* was observed only in the presence of *G. mosseae* (alone and in mixture with GF18-3). Therefore, application of the two fungi resulted in the induction of two distinct defense responses, a SA-dependent by *F. equiseti* GF18-3 and both JA- and SA- pathways by *G. mosseae* (Elsharkawy et al., 2012a).

In tomato plants, *B. pumilus* strains SE34, INR7 and T4, *B. amyloliquefaciens* strain IN937a and *B. subtilis* strain IN937b were used against CMV in a formulation supplemented with chitosan. The chitosan mixtures with IN937a, IN937b and T4 resulted in milder symptoms and the INR7 mixture in reduced viral accumulation under greenhouse conditions. The effect of these treatments against CMV was attributed to plant growth promotion as it is considered *per se* a type of resistance mechanism since virus infection in later plant developmental stages is less efficient (Murphy et al., 2003). Furthermore, the same bacterial strains (IN937a, IN937b and T4) as well as *Kluyvera cryocrescens* strain IN114 were tested in tomato cultivation against CMV, in a two-year field experiment, by Zehnder and co-workers (Zehnder et al., 2000). Application of PGPR resulted in lower disease development and increased plant growth (Zehnder et al., 2000). *B. subtilis* strain OKBHF was evaluated for its action against CMV under greenhouse conditions and its application resulted in decreased disease severity as well as promotion of growth factors such as height, fresh weight, and flower and fruit number. The molecular mechanism underlying this type of resistance was determined through gene expression analysis and it was attributed to increase levels of the three expansin genes, *LeEXP2*, *LeEXP5* and *LeEXP18* that regulate plant cell growth, and the two defense-related genes *Pti4* and *Pti6* that activate the expression of a several down-stream defense associated genes including *PR-1a* (Wang et al., 2011).

In tobacco plants, the application of *Pseudomonas chlororaphis* O6 under greenhouse conditions, reduced viral titer. Interestingly, the resistance observed was cultivar-dependent, as different tobacco cultivars exhibited distinct resistance responses upon application of O6 and infection with CMV. However, the molecular basis for these differences was not identified (Ryu et al., 2007). Another PGPR evaluated for its antiviral action against CMV in tobacco was *Paenibacillus lentimorbus* B-30488. Application of B-30488 under glasshouse conditions, decreased virulence and virus systemic accumulation. Notably, the identified molecular mechanism was linked to the restoration of gene expression and ROS production to normal levels upon treatment with B-30488 and CMV infection. Thus, in untreated plants CMV induced the expression of disease resistance protein (*NtADRI*), *PR1*, copper/zinc superoxide dismutase (*NtSOD*), catalase (*NtCAT*), *Gluc*, asparagine synthetase (*NtAsSyn*) and tetrahydrocannabinolic acid synthase (*NtTCAS*) whereas *P. lentimorbus* application, led to the stabilization and restoration of their expression in levels similar to those of uninfected plants (Kumar et al., 2016). Lastly, the application of *Penicillium simplicissimum* GP17-2 as barley grain inoculum and culture filtrate reduced disease severity and CMV accumulation in both tobacco and Arabidopsis plants. This antiviral role was attributed to increase expression of SA- (*AtPR-1*, *AtPR-2*, *AtPR-5*, *NtPR-1a*) and JA/ET- (*AtPDF1.2*, *AtHEL*, *AtVSP*, *NtCoil*) responsive genes (Elsharkawy et al., 2012b).

As mentioned above *B. amyloliquefaciens* strain IN937a was able to confer resistance against CMV in tomato in both greenhouse and field conditions. Importantly, application of the bacterial volatile produced by IN937a, 3-pentanol, in pepper plants growing under field conditions resulted, the elevated expression of SA-inducible genes *AtCaPR1* and *AtCaPR2* and enhanced protection of plants against CMV disease. Moreover, foliar application of *B. amyloliquefaciens* 5B6 and *Pseudozyma churashimaensis* strain RGJ1 in pepper plants under field conditions resulted, the reduction of both disease severity and incidence by inducing the expression of the SA- associated genes *CaPR4* and *CaPR5* (Lee and Ryu, 2016; Lee et al., 2017).

2.4.2 Viruses of genus *Potyvirus*

The *Potyvirus* genus represent one of the largest plant virus genera containing more than 162 species ([CSL STYLE ERROR: reference with no printed form.]). *Potato virus Y* (PVY) is the type member of the genus and infects a wide range of plant species mainly on the *Solanaceae* family (Scholthof et al., 2011).

In potato plants, *Bacillus vallismortis* strain EXTN-1 induced systemic resistance and reduced PVY incidence and disease severity under field conditions. EXTN-1 treated plants showed increased growth and elevated chlorophyll content even in the presence of PVY. The molecular mechanism responsible was attributed to the simultaneous induction of SA- and JA- defense pathways through the elevated expression of *StPR1* and *StPDF1.2* gene markers, respectively (Park et al., 2006).

Bean yellow mosaic virus (BYMV) and *Bean common mosaic virus* (BCMV) infect plants of the *Leguminosae* family. Currently, there are two studies in the literature for the management of these two viruses through the application of PGPR. *P. fluorescens* strain FB11 and *Rhizobium leguminosarum* by. *viceae* FBG05 were evaluated in mixture and individually for their ability to induce resistance in faba plants against BYMV under greenhouse conditions. The mixture of PGPRs resulted in elevated number of uninfected plants and in lower virus concentration in the challenged leaves of infected ones. Biochemical analysis of PGPR treated plants revealed an increased production of SA and peroxidase enzyme activity that indicated ISR against BYMV (Elbadry et al., 2006). In cowpea, application of *B. pumilus* T4 and *B. subtilis* GBO3 strains through seed treatment resulted in protection of the plants from BYMV under both screenhouse and field conditions (Udaya Shankar et al., 2009).

Zucchini yellow mosaic virus (ZYMV), *Watermelon mosaic virus* (WMV) and *Papaya ringspot virus* (PRSV) consist three of the most important threats for the cucurbit crops. The soil fungus *Beauveria bassiana* strain ATCC 74040, which is the active ingredient of the registered product Naturalis®, as well as strains ATP01, ATP05 and EABb04/01-Tip were evaluated for conferring resistance against ZYMV in squash. The application of PGPFs resulted in the reduction of disease severity, delay in symptom appearance and in increased number of asymptomatic plants. In the latter, the virus was present at the site of infection but in a very low concentration. Authors suggested that the endophytic *B. bassiana* can inhibit or interfere directly to ZYMV systemic movement through its ability to systemically move and colonize the entire plant, whereas it can simultaneously reduce virus replication and accumulation through ISR (Jaber and Salem, 2014).

The effectiveness of two PGPR strains, *B. subtilis* 281 and *B. pumilus* 293, was tested against WMV in pumpkin plants under greenhouse conditions. Drenching application of each strain separately or in combination, resulted in reduced symptom development. Furthermore, PGPR treatment resulted in lower virus concentration and enhanced growth and fresh weight of plants (Elbeshehy et al., 2015). *B. amyloliquefaciens* strain IN937a (Zehnder et al., 2000), *B. subtilis* strains 1216c and IPN-19 (Jetiyanon and Kloepper, 2002), *B. pumilus* strains SE34 and T4 (Jetiyanon and Kloepper, 2002), and *B. sphaericus* SE56 (Jetiyanon and Kloepper, 2002), already described previously, were evaluated individually or in mixture, for their antiviral activity against PRSV in squash plants under greenhouse conditions. All PGPR treatments reduced PRSV disease severity, with the mixture of IN937a and SE34, and the combination of IN937a, SE34 and SE56 inducing the highest resistance. Furthermore, different application methods, namely seed coating, root dipping and root drenching were evaluated, with root drenching showing the best results (Abdalla et al., 2017). Finally, *P. fluorescens* and *B. subtilis* strains were tested for their ability to induce resistance in leaf mustard plants against *Turnip mosaic virus* (TuMV). Application of PGPRs resulted, delay of symptom development and reduced disease severity under greenhouse conditions. The biochemical analysis of PGPR treated plants showed an increased catalase activity that was linked to the resistance exhibition (Diyansah et al., 2017).

2.4.3 Viruses of genus *Tobamovirus*

Tobamovirus genus is among the most studied in plant virology. Transmission of tobamoviruses occurs through mechanical inoculation, by contact of infected plants and through seeds. *Tobacco mosaic virus* (TMV) is the type

member of the genus and it is enlisted among the most important viruses in terms of both economical and biological impact (Scholthof et al., 2011). The capacity of rhizobacteria to control TMV was assessed in hot-pepper and tobacco plants. In hot-pepper plants, application of *Bacillus cereus*, *Bacillus* sp. and *Brevibacterium sanguinis* under greenhouse conditions resulted in a delay of symptom development and reduction of disease severity accompanied by lower virus concentration. Analysis of PGPR-treated TMV-infected plants showed increased peroxidase activity that was attributed to ISR (Damayanti et al., 2007). Moreover, Wang and co-workers (Wang et al., 2009) tested the *B. subtilis* strain G1 against TMV in tobacco plants under greenhouse conditions. Similarly, to hot-pepper experiments, delay in symptom development and reduction in TMV systemic accumulation was observed. Moreover, Wang and co-workers demonstrated that the molecular mechanism of ISR induced by G1 strain included the induction of the SA-inducible genes, *NtPR1a*, *NtPR1b*, the JA-positive regulator gene *NtCoil*, and the key defense gene *NtNPR1*. Therefore, G1 strain activated both SA- and JA- defense pathways (Wang et al., 2009).

B. amyloliquefaciens strain EXTN-1 was also tested against *Pepper mild mottle virus* (PMMoV), in tobacco plants. For the identification of the induced molecular mechanism, two tobacco cultivars were used; a susceptible and a resistant containing the *N*- resistant gene responsible for the incompatibility of the host with *tobamoviruses*. Application of EXTN-1 in the susceptible cultivar resulted in an increased number of asymptomatic plants compared to untreated ones. Interestingly, the virus accumulation in the PGPR treated plants, exhibited a pattern similar to that obtained from resistant plants challenged with PMMoV. Transcription analysis of *NtPR1a*, *NtPAL* and *3-hydroxy-3-methylglutaryl-CoA reductase* (*NtHMGR*) genes in several time-points showed increase of their expression after EXTN-1 treatment. The same expression pattern was also observed in the resistant cultivar, being different from this obtained from the susceptible-untreated control plants. The results indicated that PGPR application induced a mechanism that resembled that of the *N*-mediated resistance. To further elucidate the related defense mechanism, authors used Arabidopsis mutants exhibiting deficiency in SA-, JA- and ET- pathways and examined the transcription of the two marker genes *AtPR1* and *AtPDF1.2*. Application of EXTN-1 resulted, elevated levels of both genes implying a simultaneous activation of both SA- and JA- related response (Ahn et al.).

Cucumber green mottle mosaic virus (CGMMV) infects plants belonging to the *Cucurbitaceae* family. Drenching application of the endophytic *Stenotrophomonas maltophilia* strain HW2 in cucumber plants resulted, reduced disease index accompanied with a significant delay in virus replication and inhibition of protein expression. Transcriptional analysis of the PGPR treated plants showed more rapid and prolonged induction of the antioxidant genes *SOD* and *CAT*, and the SA-inducible *PR1* and *PR5* upon CGMMV challenge inoculation. This mechanism was the first reported to induce resistance against CGMMV (Li et al., 2016a).

2.4.4 Viruses of genus *Begomovirus*

Tomato yellow leaf curl virus (TYLCV) belongs to the *Begomovirus* genus with a single-stranded, circular DNA genome. TYLCV causes one of the most devastating diseases in tomato cultivation and it is enlisted among the regulated quarantine pests in Europe. The virus is transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner and virus management in the field is based on the regulation of its vector. The effectiveness of *Enterobacter asburiae* strain BQ9 was assessed against TYLCV in tomato plants under greenhouse conditions. The application of the PGPR resulted in the reduction of virus load that was combined with a more rapid and robust production of H_2O_2 leading to a more intense oxidative burst. The expression analysis of two marker genes *PR1a* and *PR1b* showed an enhanced defense status of the plants upon BQ9 treatment that was further increased after TYLCV inoculation. In addition, the induction of PAL, the key enzyme of the phenylpropanoid metabolism, was suggested to be involved in the restriction of the virus in BQ9-treated tomatoes (Li et al., 2016b). The second PGPR strain tested against TYLCV was *B. amyloliquefaciens* Ba13. Tomato plants treated with Ba13 and inoculated with the virus showed a delay in symptom development and low infection rate compared to the untreated plants. The Ba13 defense mechanism was further analyzed through biochemical and gene expression approaches. The bacterial treatment resulted in a rapid increase of POD, PPO, and PAL enzyme activity levels and in a rapid induction of the pathogenesis-related genes, *PR1*, *PR2* and *PR3* (Guo et al., 2019).

As described previously, the biocontrol efficacy of *B. pumilus* SE34, *K. cryocrescens* IN114, *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b strains, was tested against CMV. The same strains were also evaluated against *Tomato mottle virus* (ToMoV) another member of to the *Begomovirus* genus. Application of these PGPR in tomato plants resulted in reduced disease severity and virus incidence under field conditions. Interestingly, this effect was attributed to the reduction of the densities of viruliferous whiteflies that led to a lower dosage of ToMoV introduced in the tomato plants (Murphy et al., 2000; Zehnder et al., 2001).

2.4.5 Viruses of genus *Tospovirus*

The type member of the genus, *Tomato spotted wilt virus* (TSWV), is enlisted among the most important viruses in agriculture due to (a) its worldwide distribution, (b) its ability to infect more than 800 plant species, (c) the economic losses caused and (d) the difficulty in regulating thrips, TSWV's vectors. *P. fluorescens* strains COT-1, CHAO and COP-1 were assessed in tomato plants against TSWV. Individually or in mixture the PGPR strains resulted in reduced virus incidence under glasshouse and field conditions. Biochemical analysis of plants showed elevated levels of PAL, PPO, glucanase and chitinase enzyme activities upon PGPR application, suggesting ISR (Kandan et al., 2002, 2005). Furthermore, *P. putida* strain C13 also provided protection in field cultivated tomatoes against TSWV. Application of C13 resulted in reduced virus incidence and symptom development in fruits accompanied with enhanced growth (Zaccardelli et al., 2011). Although the biocontrol of TSWV by *Pseudomonas* sp. was attributed to the ISR, the molecular mechanism was not fully elucidated.

Finally, Abdalla and co-workers (Abdalla et al., 2017) have demonstrated that PGPR strains used for the management of PRSV in squash (described in the *Potyvirus* section) were also effective in tomato plants against *Tomato chlorotic spot virus* (TCSV) another member of the *Tospovirus* genus as drenching application resulted in decreased disease severity (Abdalla et al., 2017).

2.4.6 Banana bunchy top virus

Banana bunchy top disease caused by *Banana bunchy top virus* (BBTV) is the most serious virus disease in banana-growing regions worldwide. BBTV is the type member of the *Babuvirus* genus. Kavino and co-workers (Kavino et al., 2008) tested *P. fluorescens* strain CHAO supplemented with chitin against BBTV. The application resulted, reduction of disease incidence under both field and greenhouse conditions. The related mechanism was linked to the increased accumulation of oxidative enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), pathogenesis-related (PR) proteins, chitinase, β -1,3-glucanase and phenolics (Kavino et al., 2008). Furthermore, *Pseudomonas* sp. strain mixtures, EPB5 / EPB22 / Pf1 / CHA0, EPB5 / Pf1 / CHA0 and EPB22 / Pf1 / CHA0 provided more than 60% protection of banana plantlets under field conditions. This effect was also linked to the increased production of peroxidase, polyphenol oxidase, PAL PR proteins (Harish et al., 2008, 2009).

2.4.7 Potato virus X

Potato virus X (PVX) is the type member of the *Potexvirus* genus, widely distributed, and transmitted by contact. *B. vallismortis* strain EXTN-1 (*Potyvirus* section) was evaluated for its ability to induce resistance in potato plants against PVX. Results in field trials showed an effect against PVX of EXTN-1 similar to that observed against PVY. *B. vallismortis* strain EXTN-1 treatment resulted in reduced PVX disease severity. The authors suggested that the elevated expression of both the SA- and JA- pathways resulted in the induction of a common defense mechanism that could confer resistance against both PVY and PVX, two viruses belonging in two distinct genera (Park et al., 2006).

2.4.8 Tobacco streak virus

Tobacco streak virus (TSV), genus *Ilarvirus*, is distributed worldwide and is transmitted in nature through thrip vectors and seeds. TSV infects a wide range of plant species and it is the causative agent of cotton necrosis disease. Vinodkumar et al., (2018) evaluated *B. amyloliquefaciens* VB7 strain against TSV in cowpea plants in field conditions. Drenching and foliar application of VB7 resulted in reduction of disease incidence whereas the antiviral activity of the VB7 was further supported by the reduction of virus concentration in the treated plants. Authors also suggested that the bacterial strain provided effective resistance to the plants through the promotion of plant growth, ISR and putatively by the production of peptides exhibiting antiviral activity (Vinodkumar et al., 2018).

2.5 Antiviral activity of *Bacillus amyloliquefaciens* strain MBI600

Most publications of antiviral action of PGPMs are based on laboratory bacterial strains. In one of the rare studies with commercial bio-pesticides, the antiviral activity of *B. amyloliquefaciens* strain MBI600, active ingredient of the bio-fungicide Serifel® (BASF, SE) was tested against TSWV and PVY in tomato plants (Beris et al., 2018). Three application schemes were tested against TSWV which consisted of triple drench or triple foliar applications with seven days interval, and single application in dry potting medium prior seed sowing. Experiments were conducted under both

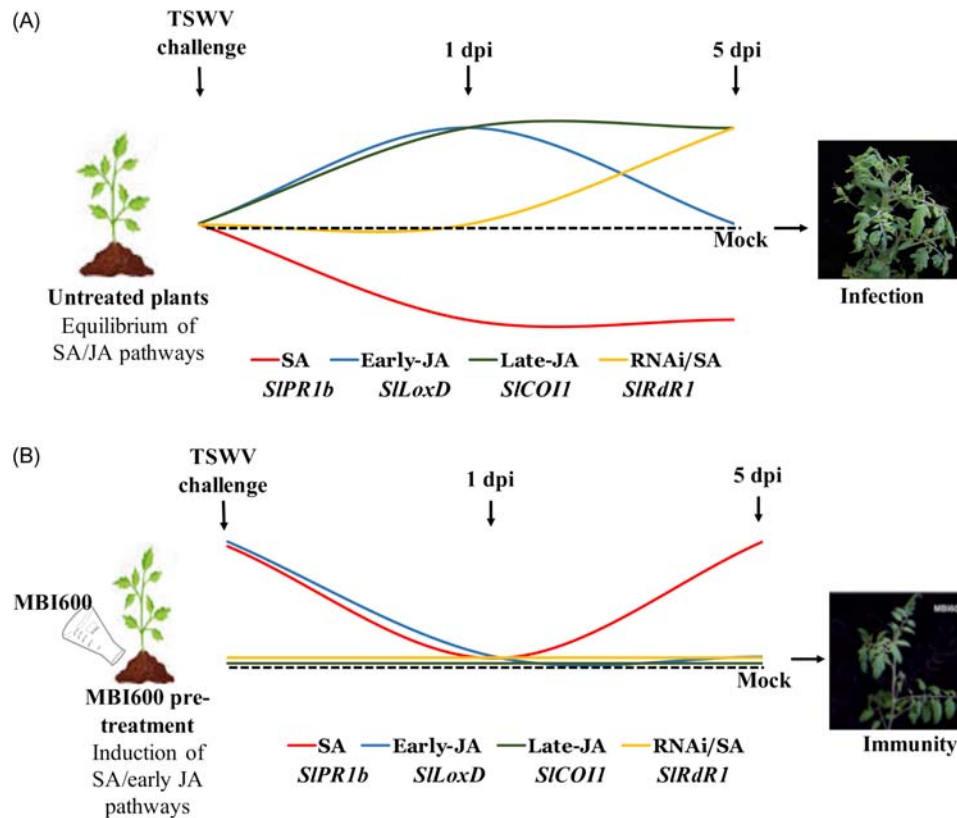


FIGURE 2.2 Schematic representation of the progression of the relative expression of the examined genes related to SA- (*pathogenesis-related protein 1*, *SIPR1b*), early (*Lipoxygenase D*, *SiLoxD*) and late JA- (*Coronatin insensitive 1*, *SICO11*) pathways and to the basal antiviral defense (*RNA-dependent-RNA-polymerase 1*, after TSWV infection in control (A) and MBI600 treated plants (B). (A) TSWV infection resulted in the disruption of the SA/JA equilibrium and to the induction of the JA-pathway for the establishment of a successful TSWV infection. (B) MBI600 treatment resulted in a primed defense status of plants that prevented the TSWV hormonal manipulation at 1 dpi, whereas MBI600 led to further induction of the SA-mechanism (5 dpi). It is plausible that the shift caused by MBI600 between the SA and JA defense mechanisms resulted in the protection of tomato plants against TSWV. The expression profile of all biological groups was compared to rub-inoculated, uninfected plants (Mock) (dpi: days post inoculation). Based on gene expression analysis described in (Beris et al., 2018).

greenhouse and growth chamber conditions. All MBI600 applications resulted in significant reduction of TSWV incidence. Interestingly, the exhibited resistance had the form of immunity as virus was not detected neither in virus-challenge inoculated leaves of MBI600 treated plants. However, against PVY the MBI600 treatment resulted in a seven days delay in systemic detection of the virus which was followed by an equal delay in symptoms development.

To get insight into the molecular mechanism underlying the antiviral activity of MBI600, an expression analysis was conducted just before, and at several timepoints after virus challenge, targeting genes of the SA- (*SIPR1b*), early (*Lipoxygenase D*, *SiLoxD*) and late (*Coronatin insensitive 1*, *SICO11*) JA-pathways, the key regulator defense gene *Non-expressor of PR genes* (*SINPR1*), the basal antiviral defense (*RNA-dependent-RNA-polymerase 1*, *SIRdRp1*) and priming (*Mitogen-activated Protein Kinase 3*, *SIMPK3*). The analysis of MBI600 treated plants, prior virus challenge inoculation showed a strong induction of the SA-related genes *SIPR1b*, *SIMPK3*, and *SINPR1*. Moreover, elevated level of *SiLoxD* indicated induction of the JA-response which however was not confirmed by an induction of the second JA-associated gene *SICO11*. Further analysis of the TSWV-challenged leaves at one- and five-days post inoculation (dpi) and of the upper non-inoculated leaves of PVY-challenged plants at one, two and 5 dpi led to the hypothesis for MBI600 mode of action proposed below (Fig. 2.2). As illustrated in Fig. 2.2A, in untreated control plants TSWV inoculation resulted in the disruption of the equilibrium between the SA and JA pathways, which was characterized by the induction of the JA-dependent defense mechanism in the expense of the SA-one. In accordance to the literature (Abe et al., 2012), the gene expression analysis revealed that TSWV downregulated the important in antiviral defense SA-pathway in order to establish a successful infection. In contrast, pretreatment of plants with MBI600 resulted in a primed defense status characterized by elevated levels of the SA-inducible defense genes, which despite the challenge inoculation with TSWV, resembled those of healthy uninfected plants (one dpi). It is plausible that this primed status

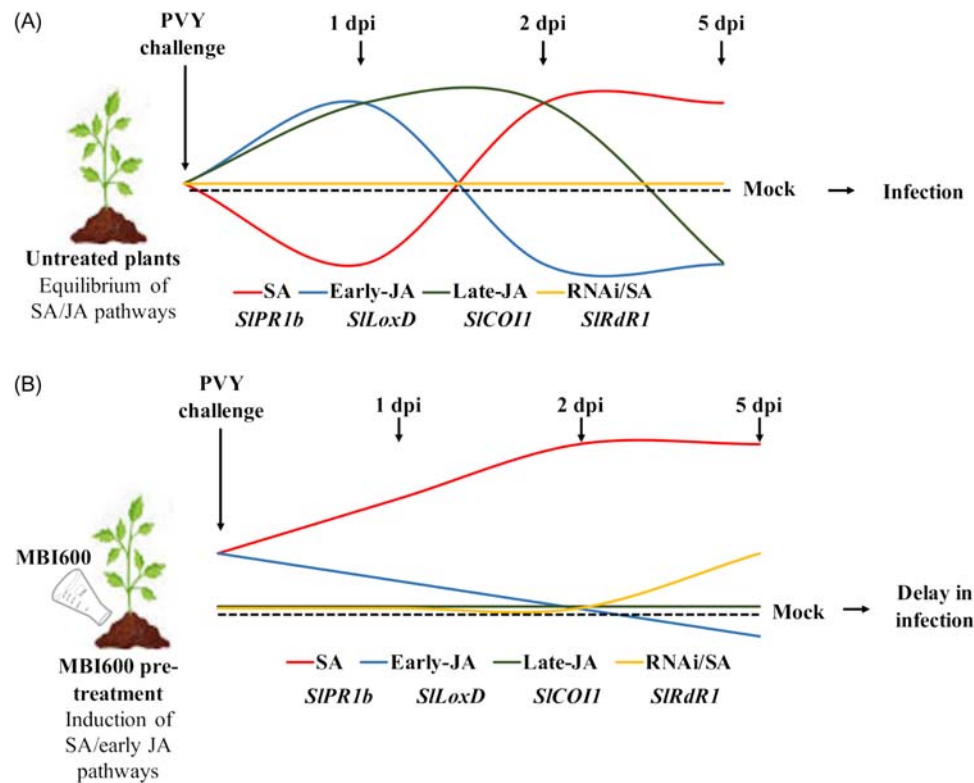


FIGURE 2.3 Schematic representation of the progression of the relative expression of the examined genes related to SA- (pathogenesis-related protein 1, *SIPR1b*), early (Lipoxygenase D, *SILoxD*) and late JA-(Coronatine insensitive 1, *SICO11*) pathways and to the basal antiviral defense (RNA-dependent-RNA-polymerase 1), after PVY infection in control (A) and MBI600 treated plants (B). (A) PVY infection leads to the induction of JA pathway and downregulation of the SA one at one dpi. The SA/JA balance is then inverted at two and five dpi and this pattern leads to the successful PVY systemic infection. (B) In MBI600 treated plants an upregulation of the SA-response mechanism at all time-points checked in combination with a more rapid induction of the RNAi defense mechanism was recorded. The latter may have as result the delay of PVY systemic infection. The expression profile of all biological groups was compared to rub-inoculated, uninfected plants (Mock) (dpi: days post inoculation). Based on gene expression analysis described in (Beris et al., 2018).

prevented the manipulation of the plant hormonal pathways by TSWV at the initial stages of infection, resulting in failure of TSWV infection. A further induction of the SA-pathway at five dpi can be attributed to the sequential MBI600 applications retaining active the SA-associated defense mechanism (Fig. 2.2B).

In the case of PVY, a more complex expression profile was obtained from the analysis in the upper non-inoculated leaves at one, two and five dpi. More precisely, in untreated-control plants at one dpi, PVY downregulated the SA and upregulated the JA pathway, a situation that was reverted at later stages of infection (two and five dpi) (Fig. 2.3A). In MBI600 pre-treated plants, the SA-pathway remained upregulated and the JA pathway downregulated during all time-points tested. Furthermore, the increase of *SIRdR1* levels at 5 dpi, indicates a more rapid induction of RNAi in the MBI600 plants compared to untreated ones. Thus, the activation of the SA-dependent pathway combined with the induction of the RNAi basal defense observed in the MBI600-treated plants may result in the delay of PVY systemic infection (Fig. 2.3B).

In conclusion, the results demonstrated a clear but not uniform antiviral function of MBI600 against TSWV and PVY in tomato plants. This is based mainly on the induction of ISR and the SA-mediated defense response. The distinct outcome in the level of the protection conferred by MBI600 may reflect the differential manipulation of the hormonal pathways by the two viruses (Beris et al., 2018).

2.6 Conclusions

Over the past decades, an increasing number of studies focused on the use of PGPM against plant pathogens. PGPM's ability to induce resistance against both biotic and abiotic stress, as well as to promote plant growth, had rendered them a promising eco-friendly approach for the control of plant diseases. Hence, several PGPM have been registered as

bio-control agents against fungal and bacterial pathogens. In the studies presented in this chapter, PGPMs also provided different levels of protection in various hosts against a range of viruses from different species and genera. In total, PGPMs were tested in eight host species against 18 different viruses, belonging to eight genera, with different strategies of genomic organization, multiplication, means of transmission and infection processes. In the majority of the experiments, PGPM's application was carried out in the field or into greenhouses and resulted in reduced disease severity or more importantly disease incidence. Moreover, the underlying resistance mechanism was attributed primarily to the induction of the SA-pathway, or to the induction of both SA and JA defense signaling pathways. There are accumulating pieces of evidence that PGPMs induce a resistance mechanism which is compatible with the model of the enhancement of antiviral resistance of susceptible plants after exogenous application of SA. Several characteristics of this model remain to be illuminated, however it has been proposed that SA enhances the RNA-silencing antiviral defense of plants through the induction of RdR1 and the triggering of several redundant pathways [reviewed in Alazem and Lin (2015)]. The activation of JA-pathway is important for plant defense against various pathogens or pests, however its role in antiviral defense is contradictory. Nonetheless, it might be a desirable PGPM feature due to its action against virus insect vectors [reviewed in Rashid and Chung (2017)].

It is evident that the application of PGPMs resulted in an antiviral effect only when it was performed before the occurrence of virus infection and it does not provide a therapeutic action. Moreover, the PGPM's antiviral action was not uniform against the different virus species tested; a variety of factors could be responsible for the latter, such as the genetic background of the host and the virus, the defense mechanism induced in the host, the counter-defense strategy of the virus, the environmental conditions and the developmental stage of the plants. Therefore, the antiviral PGPM's effect should be examined on a case-by-case basis including field trials in a variety of environmental conditions and considering vector transmission. These requirements may explain the lack of PGPMs registered against virus diseases. Nonetheless, taking into account the limited resources for the control of viral diseases, it is important to continue the research for the identification of PGPMs either new or already registered against fungal or bacteria pathogens, which could provide protection against viruses.

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Further reading

ICTV Master Species List 2015 v1.

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Chapter 3

Multilegume biofertilizer: a dream

Ikbal^{1,#}, Nishat Passricha^{2,#}, Shabnam K. Saifi², Virendra K Sikka¹ and Narendra Tuteja²

¹Department of Molecular Biology Biotechnology Bioinformatics, College of Basic sciences, CCS-Haryana Agricultural University, Hisar, India,

²Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

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3.1 Introduction

Biological nitrogen fixation plays a critical role in sustainable agriculture, because it reduces the need for exogenous fertilizer while providing carbon, nitrogen, phosphorus and other nutrients for producing protein rich foods. Therefore, the agriculture system excessively depends on fertilizer application. Nitrogen is one of the essential common nutrient elements for restraining crop yield (Zhao et al., 2005). It is an imperative growth element, due to its involvement in synthesis of major biological macromolecules such as nucleic acids, amino acids, lipoproteins, glycoproteins etc. (Mosier et al., 2004). Although our atmosphere is plentiful of nitrogen, but living beings cannot utilize it. To utilize it in biological process it should be fixed by the involvement of special microorganisms. Hence the agriculture systems adopt chemical fertilizers (Galloway et al., 2008). Despite of the fact that introduction of chemical fertilizers led to significant increase in crop yields nonetheless it pollutes the environment especially soil and water. As a consequence of massive use of chemical fertilizers, the unremitting energy dissipation results in upmarket fertilizer value. Therefore, effective utilization of natural resources, e.g. atmospheric nitrogen is imperative to maximize the yield. Biological nitrogen fixation (BNF) is a renewable source of nitrogen that converts atmospheric di-nitrogen (N₂) into plant usable ammonia through biological process (Ibanez et al., 2017). Nitrogen fixation and plant growth enhancement by rhizosphere bacteria might be important factors for achieving sustainable agriculture (Dixon and Kahn, 2004; Mohammadi et al., 2012). Most of biological nitrogen fixation eminently carried out through association between rhizobia and leguminous plant. The enzymatic reduction of nitrogen to ammonia, replenishes the loss of nitrogen within the natural succession (Hardson and Atkins, 2003). This process also increases organic matter, stabilize the soil and enhance nitrogen transfer from N₂ fixing to non N₂ fixing species associated with soil-plant ecosystem. Biological nitrogen fixation is important for agriculture because it allocate significant amount of nitrogen to total nitrogen budget in the ecosystem and reduces the requirements for synthetic fertilizers during the growth of leguminous crops (Singh et al., 2008; Shetta et al., 2011).

Most of the *Rhizobium* species specifically interact with a particular leguminous plant and out-turn the formation of effective nitrogen fixing nodules while many plant species share common *Rhizobium* for nitrogen fixation. The degree of host specificity varies tremendously among rhizobia (Li et al., 2011). These are the clues which indicate that rhizobia

#. Equal authorship

could infect non host legumes. Genetic exchange among heterologous *Rhizobium* is one of the perspective which leads to broaden the host range (Young and Johnston, 1989; Vlassak and Vanderleyden, 1997). These types of promiscuous rhizobia could be used as multilegume biofertilizer. It could be more beneficial for the agricultural practices because selection of correct *Rhizobium* strain for a particular leguminous plant species is difficult. Most of the commercially accessible *Rhizobium* inoculants are specific with narrow host range. The multilegume Biofertilizer having high nitrogen fixation ability could achieve substantial increases in legume nodulation, biomass yield, and post-crop soil nitrate levels. Development of superior and effective biofertilizer is a common approach to improve the symbiotic nitrogen fixation and crop productivity. It could exhibit a great contribution to the cropping system. Soils contains adequate populations of rhizobia, the communities are diverse and become less effective at nitrogen fixing. The application of such multilegume biofertilizer can successfully compete with established soil rhizobia.

3.2 Biofertilizer for nitrogen fixation

Rhizobium species have been successfully used worldwide as a biofertilizer leading to effective establishment of N₂-fixing symbiotic association with leguminous crop plants (Shiferaw et al., 2004, Miller et al., 2007). BNF oversee more than 175 million tons of the world's annual nitrogen sources (Chafi and Bensoltane, 2003). Legumes with *Rhizobium* species biologically fix 195 Tg nitrogen year⁻¹ (Vitousek et al., 2013). The ecological importance of nitrogen fixation in legumes through symbiosis has been extensively investigated and documented. The establishment of symbiosis involves a complex series of events starting from exudation of flavonoids by the plant. These compounds are continuously secreted by plants, but their concentration increase when appropriate rhizobia identified in rhizosphere (Hassan and Mathesius, 2012). The signal exchange between the two partners varies from highly specific to highly promiscuous (Hirsch and Fujishige, 2012). Rhizobia colonize intracellularly on roots of host plants and induce the emergence of organs called nodules. In some incompatible partners, the initial signal exchange takes place, nodules may be obtained, but *Rhizobium* does not release from infection thread and effective symbiosis does not take place (Miller et al., 2007). In the *Rhizobium*-legume symbiosis, the bacterial partner differentiates into non-dividing endo-cellular symbionts. These symbionts fix atmospheric N₂ into NH₃/NH₄⁺ through the induction of nitrogenase complex that is consumed by host plant (Patriarca et al., 2002). Several previous studies were performed on *Rhizobium* species which dealt with the strain diversity, phylogeny, and mechanisms of host specificity, but these are not yet fully addressed.

3.3 Diversity of rhizobia fraternize legume

Biological nitrogen fixation reflects the activity of rhizobia which establish a mutualistic symbiosis with leguminous plants. Presently rhizobia consist of 61 species which belongs to 13 genera, namely *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Allorhizobium*, *Methylobacterium*, *Phyllobacterium*, *Ochrobactrum*, *Herbaspirillum*, *Burkholdera*, *Cupriavidus*, *Devosia*. Host specificity is an important characteristic of symbiotic interaction, which classifies the rhizobia species to commence symbiosis with specific legumes (Ampomah et al., 2008). But, identification of new strain has complicated the situation and symbiosis became more diverse than previously presumed. Some promiscuous rhizobia make symbiotic association with wide range of legumes and capable in effective nitrogen fixation. These diverse organisms have extended our area of research outside the host specificity. However, predicting which regulatory factor is most relevant for effective symbiosis in diverse taxa is more challenging. For example, there are evidences that variation in nod factors, specify the host for a particular *Rhizobium* but *Sinorhizobium* sp. NGR234 and *Sinorhizobium fredii* USDA257 nodulates 112 and 77 legumes respectively without any variation. How these variations come up with broad diversity of recently found microorganisms is not known.

3.4 Symbiotic association mechanism

The establishment of symbiosis need successful entry of *Rhizobium* to legume roots. It is multifaceted process driven by microsymbiont but eventually controlled by host plant (Murray, 2011). Plant root exudates (flavonoids) attract rhizobia by positive chemotaxis at localized site (De-la-Pena et al., 2008, 2010; Badri et al., 2009; Badri and Vivanco, 2009). These flavonoids bind to bacterial protein to elicit chemo-static reaction and act as strong or weak inducer for different kinds of nitrogen fixing genes, including *nod* genes (Ryu et al., 2012). Cytokinins are essential phytohormones for rhizobia infection and nodule development, particularly for initiation of root cortical cell divisions and induction of critical gene expression in the NF-signaling pathway (Jianping et al., 2018). The phytohormone auxin is essential for cell divisions in plants and required for both indeterminate or determinate nodule development (Suzaki et al., 2012).

Plant infection starts with the attachment of rhizobia to the tips of root hairs and is followed by formation of “shepherd’s crook” within which microsymbiote established, forming an infection pocket (Oldroyd and Downie, 2004; Fournier et al., 2008). This organelle-like structure containing the bacteroid is called the symbiosome (Brewin, 2004). The nod factor perception in legume roots induces expression of nodulation genes and causes development of a new organ, a nodule. Thus, a complex, regulated association takes place under the coordinated expression of several symbiosis-related genes in both the plant and the bacteria (D’Haeze and Holsters, 2002).

However, a group of rhizobia maneuver a different pathway to commence symbioses with leguminous plant, where purine derivatives play a major role to initiate nodule formation. The universality of the nod factor (NF) paradigm was recently overturned by showing that the genomes of some bacteria that elicit root and stem-nodules on a particular group of plants lack the canonical *nodABC* genes required for the synthesis of the nod factor (Giraud, 2007). This evidences that, some rhizobia ply nod factor independent strategy to associate with plants. Certain rhizobia invade plant root through ‘crack entry’ (intercellular root) but in that case, no infection thread formation takes place inside root hairs (Madsen et al., 2010). These finding indicates presence of alternative pathway that facilitates the entry of the *Rhizobium* in to the roots of diverse legumes.

3.5 Nod factors and their regulation in symbiotic infection

The *Rhizobium*–legume symbiosis, compatible partners recognize each other through an exchange of signals. Almost, all *Rhizobium* species have common *nod* genes (*nod A*, *B*, and *C*) which are responsible for chitin backbone (nod factor) synthesis. These factors are key compounds mediating communication and biological interaction between symbiosis partners. Host specific *nod* genes supervise various modifications in chitin backbone and confer the specificity of *Rhizobium* for a particular host (Gibson et al., 2008). Therefore, it is possible to assume that the host-rhizobia co-evolution involved modifications of an ancestral nod factor structure. The expression of *nod* genes in response to host plant flavonoids appears to be positively regulated by two pathways; first involves NodD1 which is a common regulatory protein to almost all rhizobia, second involves NodVW which regulate the expression of other *nod* genes. Despite these factors allow rhizobia to infect their hosts but these are not sufficient to confer nodulation, there are additional factors probably acts within the plant (D’Haeze et al., 1998). Thus, a number of metabolites synthesize in response to nod factor when rhizobia comes in contact with plant roots (D’Haeze and Holsters, 2002; Gibson et al., 2008). The composition and concentration of nod factor excreted by rhizobia are important in the rhizosphere for effective symbiosis (Relic et al., 1994).

The association of rhizobia and leguminous plant is complex, coordinated and specific. The host range determined at early stages of the plant-bacterium interaction (Walker et al., 2003). Some of the essential *nod* genes and *hsn* genes confer host specificity by modifying the core structure of the oligosaccharide backbone (Spaink et al., 1995). Thus, although the nod factors are essential to nodulation and their modification may contribute to host-specificity, but several other elements also exist which specifying the host range at various levels (Jianping et al., 2018). In few wild legume rhizobia specificity exists but most of them are promiscuous (Zahran, 2001). These possibilities contradict the dogma of nod factor that decide host specificity. There are examples to prove the promiscuous nature of some rhizobia. Although the major nod factor secreted by *Rhizobium leguminosarum* bv. *Trifolii* and *Rhizobium leguminosarum* bv. *viciae* are identical yet these bacteria interact with different host (Spaink et al., 1995). Similarly, *Rhizobium meli* and *Mesorhizobium loti* produced same nod factor but the two species infect distinct host (Cardenas et al., 1995). In contrast different rhizobia nodulate the same host may produce different nod factors (Poupot et al., 1995). It is possible that during interaction between rhizobia and its host some other modulator overlooked the major nod factor. Based on the information of genetic resources of model leguminous plant, more than 40 genes have been found essential for *Rhizobium* endosymbiosis (Sandal et al., 2006; Oldroyd and Downie 2008). The common *nodC* gene is essential for symbiosis in all rhizobia examined so far. It codes for N-acetyl-glucosaminyl-transferase which entail the first step of nod factor synthesis and also determines the host range (Perret et al., 2000). The outcomes of previous studies concluded that length of chitin backbone strenuously contributes to the host specificity and influence the activities of host plants (Eric et al., 1997). However, one part of the host specificity is regulated on the other level at which NodA recognizes acyl chains and transfers them to nod factor, as a final structure that is specific for the host plant (Jelena et al., 2010). One report manifested that *nodA* is crucial for synthesis of host specific lipo-chitoooligosaccharides in *Rhizobium leguminosarum* bv. *Viciae* (Ritsema et al., 1996). These findings, suggested that not only the host specific nod factor but the common nod proteins also participated in optimal production of host specific LCOs.

There is a considerable population of bacteria that are similar to rhizobia but does not have common *nod* genes but still they are involved in effective symbiosis (Melicent et al., 2006). The variation in the host range which is mediated

by *nod* genes, relies on two different genetic process, non-allelic variability by using different combinations of genes, and allelic variation within genes (Denarie et al., 1992). The flavonoids produced by plants activate the transcription of *nodD* genes (Spaink et al., 1987). NodD shows certain flavonoid specificity that restricts *nod* gene induction (Spaink, 1996). There are evidences that host specificity resolved at initial stage of regulational level by *nodD* gene (Beatrice et al., 1987). Some species specific *nod* genes e.g. *nodE* append variability in nod factor that have different properties in different *Rhizobium* species. NodE product is also one of the factors that distinguish host range of nodulation. It has been found that *nodE* gene is important for specific nodulation in *Rhizobium trifolii* and *Rhizobium leguminosarum* (Herman et al., 1989). It participate in the modification of common nod factor and mutation within these genes leads to ineffective nodulation. In contrast mutation within common *nod* genes has strapping effect on nodule formation. In one of the report it was noted that *nodE* mutants of *R. meliloti* secreted modified nod-factors, but these mutants still able to infect various *Medicago cultivars* (Ardourel et al., 1994). The *nodW* is also a key component in regulation of *nod* gene expression which has been proved in *Bradyrhizobium japonicum* (Sanjuan et al., 1994). The significance of NodV in phosphorylation of NodW has been described by Gottfert et al. (1990). Their importance for effective nodulation of alternative host plant could be elucidating by the possibility that leguminous plant excrete inducer which specifically interact with these factors not NodD1. Mutations in these genes abolish the potential of *Bradyrhizobium japonicum* to infect different legumes (Gottfert et al., 1990). From these observations it is now clear that various *nod* genes participate in host range determination.

The universality of nod factor archetype is now overturned with the findings of microsymbionts that lack the canonical *nodABC* genes which are essential for synthesis of nod factor core (lipo-chito-oligosaccharide). Although *nod* lacking bradyrhizobia only nodulate few subset of *Aeschynomene* plants while bradyrhizobia containing *nod* genes have wider host range (Giraud and Fleischman, 2004). *Bradyrhizobium* strain BTAi1 and ORS278 trigger stem and root nodules on *Aeschynomene* plant though they omit common *nodABC* genes (Giraud, 2007). Comparative genome analysis of three *Frankia* also confirmed the lack of nod factor genes but still they performed nitrogen fixation (Normand, 2007). These studies indicate that such type of rhizobia uses nod factor independent approach to enter into symbiosis. Exploration of these types of bacterial biodiversity could be advantageous to the discovery of novel microsymbionts in which nod factor are not involved (Catherine et al., 2009).

Analysis of different pathway led to the conclusion that, symbiotic association does not depend on single pathway instead they evolved several approaches to enter into host plant (Ibanez and Fabra, 2011). Some *Rhizobium* species excrete Nops (nodulation outer proteins) that are participated in symbiosis through type III secretion system for nodule formation (Fauvart and Michiels, 2008; Deakin and Broughton, 2009). These proteins suppress the immune response of host plant or modify the root cell cytoskeletal during nodule formation. Okazaki et al. (2013) observed the hijacking of leguminous nodulation signaling by the rhizobial type III secretion system and found that the interaction does not require the initial phase of molecular interaction. Intriguingly, the *nodC* mutant were not as much effective as the wild type *B. elkanii* indicating nod factor still important in most of the *Rhizobium* for effective symbiosis.

3.6 Biotechnological applications for the development of multilegume biofertilizer

Earlier, developing countries were not well equipped for biofertilizer production, storage and distribution, so they were reliant upon developed countries. Although some developed countries have healthy economic status and satisfactory environmental conditions but, use of biofertilizer is still shorter (Kakraliya et al., 2018). But now, advancement of biotechnological tools makes it possible to develop improved strains for their crops. The rational combination of biofertilizer and N chemical fertilizer can be effective for profitable and sustainable crop production (Botir and Anvar, 2018). Biofertilizer combined with organic fertilizer combination produced higher bacterial population than inorganic fertilizer combination (Suliasih and Widawati, 2018). The nature harbor diverse rhizobia whose efficient management and conservation become possible through biotechnological tools. The utilization of wild legume rhizobia could be beneficial for agriculture system because they have high nitrogen fixing activity, promiscuous nature and tolerance to unfavorable conditions (Zahran, 2001). Wild types rhizobia are good aspirants to understand the regulation of nod factor independent pathway of symbiosis. These effective rhizobia with interesting characteristics could be used as multilegume biofertilizer for inoculation of cultivated legume crops.

There are considerable possibilities to improve rhizobia for the enhancement of N₂ fixation through genetic engineering. This could be obtained through manipulation of both rhizobia as well as host plant genotype. The genetic events applied for symbiotic improvement could flare a new light on the evolutionary history of rhizobia. Further use of biotechnological methods could make it possible to investigate the other factors involves in the process of nitrogen-fixing *Rhizobium*-legume symbiosis. These factors affect the competitiveness, establishment and efficiency of rhizobia.

The complex nature of cross-nodulation relationships between diverse rhizobial strains and legume hosts is already highlighted.

The host range diapason of *Rhizobium* species has been a matter of extensive investigation. It has been already proved that rhizobia can make symbiosis with nonhost leguminous plant. The first and probably the most detailed report by Wilson (1939), who found two *Rhizobium* strains which can nodulate more than 25 plant species and having extremely promiscuous symbiotic properties. Similarly, Hernandez et al. (1995) also defined the host ranges of *Rhizobium etli* and *R. Tropici* and found that all the strains have broad host ranges that infect several legumes including woody species such as *G. maculata* and *L. leucocephala*. In direction of host range study, several other researchers execute infection assay with different leguminous plants and illustrated that promiscuous rhizobia have the ability to infect several legumes, while non-promiscuous rhizobia need specific interaction for symbiosis (Bertrand et al., 1985; Pueppke and Broughton, 1999; Radhey et al., 2005). These types of rhizobia could be utilized for production of multilegume biofertilizer. Thus promiscuity appears a matter of consideration for tropical legumes, such out-of-group nodulations, however, are often ineffective (Martinez and Caballero, 1996; Bala et al., 2003). One possible alternative to this problem resides on the use of free-living bacteria, such as *Pseudomonas*, presenting high levels of ACC deaminase activity that may be used as adjuvants in the nodulation process by decreasing inhibitory ethylene levels (Francisco et al., 2019). Effectiveness is prerequisite for multilegume biofertilizer so that it could be better approach to enhance biological nitrogen fixation in legume crops (Ojo, 2001; Setiyo, 2011).

3.7 Improvement of complex carbohydrate utilizing ability

The process of rhizobial infection into legume roots has increasingly been associated with complex carbohydrate degrading enzymes (Turgeon and Bauer, 1985). Electron microscopic studies of nodulation process have shown localized deterioration of root hair cell wall which indicates the role of cell wall degrading hydrolytic enzymes in symbiosis (Higashi and Abe, 1980; Callaham and Torrey, 1981; Ridge and Rolfe, 1985). *Rhizobium* species utilizes different carbohydrate sources as these are capable of producing important enzymes like amylase, pectinase and cellulase for adoption or establishment of plant microbe interaction (Emtiaz et al., 2007; Shetta et al., 2011). These enzymes involved in breaking of intracellular bonds and loosening of cell wall during symbiosis (Hussain et al., 1995). The cells thus release in the rhizosphere and consequently increase the bacterial colonization towards roots. Likewise, Kennedy and Islam (2001) exploited 2,4-D (2,4-dichlorophenoxy acetic acid) to remove one of the barriers to symbiosis. It causes damage to the root cells and facilitates the entry of the bacterium into the roots of many legumes. This is one of approach used by other investigators to ease bacteria which produce cellulase and polygalacturonase to induce effective nodulation (Azam et al., 2005). The effectiveness of *Rhizobium* could be improved by increasing their hydrolytic enzyme activity. Mutation is one of the strategies though which the hydrolytic enzyme activity of *Rhizobium* could be improved. The fast growing *Rhizobium* which utilized a wider range of carbon compounds performed better than the slow growing (Stowers and Eaglesham, 1984; Hafeez et al., 1995). These studies are consistent with earlier reports of rhizobia produced enzymes that can break glycosidic bonds present in plant cell walls and provide evidence that these enzyme activities are cell bound. *Klebsiella pneumonia* was found effective in hydrolyzing α -linked glucan of starches, pectins and also splitting 1,4- β -glucosidic bonds of cellulosic components and proved as effective nitrogen fixer having broad host range (Derrick et al., 2008). Ikbali et al. (2016), were able to improve the hydrolytic enzyme activity through gamma rays and developed mutant strains which significantly increased the number of nodules and nitrogen fixation over the control strains. Rhizobia having high hydrolytic enzyme activity growing better on CMC and/or pectin were behaving as super-nodulating rhizobia (Aggarwal et al., 2000). Conceivably cellulase producing strain *Pseudomonas trivialis* 3Re27 along with *Rhizobium* was found to significantly enhance symbiotic effectiveness and nitrogen fixation with co-inoculated plants (Egamberdieva et al., 2010). The reason for enhancement of nodulation in legumes might be the hydrolytic enzymes (cellulase). However, the mechanism behind this conception is only partly accepted. The cell-bound cellulase enzyme from *Rhizobium* could degrade the tip of root hairs wall, make a canyon to allow penetration of bacterial cell and establish microsymbiont to develop nitrogen fixing nodules (Robledo et al., 2008). Such microorganisms suppress plant immunity and behave like parasite rather than legume symbionts (Joaquin et al., 2018). Previous studies also indicate that *Rhizobium* produce hydrolytic enzymes capable of degrading cell wall polymer of host plant (Verma et al., 1978; Angle, 1986), but little was known about their molecular mechanism. Collectively these investigations exculpate a complimentary role of cellulose along with nod factor in boosting root hair infection at early stage.

3.8 Improvement of symbiotic efficiency of rhizobia through genetic modification

A wide range of molecular genetics and genome editing techniques (e.g., Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR) are now offering unprecedented opportunities to create genetic variations and manipulate the biological system to produce improved strains. Understanding the processes that drive nodulation will provide genetic targets that can be used for selecting superior legume cultivars and rhizobia strains that are competitive, highly effective and stress tolerant (Brett et al., 2019). Genetic transfer of symbiotically favorable determinants is an alternative approach to construct improved *Rhizobium* strains. Although microsymbionts are easy to manipulate through conjugation or recombinant DNA technology, but the host plants could also be modified to improve symbiosis and nitrogen fixation. In view of microbial genetics, there are three approaches of genetic variation in bacteria: (1) Mutation- alteration of the nucleotide sequence, (2) Intragenomic rearrangement of DNA sequences (3) Transformation- uptake of foreign DNA (Arber 2000). The application of such approaches can be useful for improvement of inoculants with effective symbiotic nitrogen fixation. However, ignorance of improved protocols of bio-fertilizers application to the field is one of the limiting factors to bio-fertilizers efficiency (Itelima et al., 2018).

The *nod* genes could be interchangeable between different *Rhizobium* species. Nodulation and the levels of nitrogen fixation can be significantly higher when plant is infected with *Rhizobium* containing the hybrid *nod* gene, which activates the transcription of other genes independent from flavonoids (Schlaman et al., 1991; Spaink et al., 1991). Plasmid transfer in *Rhizobium* may increase nodulation and nitrogen fixation (De Jonj et al., 1982), and there is one report of a plasmid loss that improves symbiotic properties in *Rhizobium loti* (Pankhurst et al., 1986). Martinez et al. (1987) demonstrated that gene transfer between *Rhizobium* species has led to improve symbiosis along with growth and development of plant. Transfer of *nodD1* from NGR234 into *Rhizobium leguminosarum* bv. *Trifolii* through conjugation extends its host range to *Parasponia andersonii* (non legume) which is a host of NGR234 (Spaink et al., 1987). Esperanza and Monica (1990) genetically modified the *Rhizobium* strains to increase nodulation competitiveness in bean (*P. vulgaris* L.). Truchet et al. (1991) developed trans-conjugants from di-parental mating and found significant increase in nodule number and plant dry weight over their mid-parents. Similarly Jabbouri et al. (1998) transferred *nolO* into *R. fredii* through conjugation and reported host range extension of the recipient to infect non-host *Calopogonium caeruleum* and *Lablab purpureus*. Large fragments of the symbiotic plasmid of *Rhizobium* sp. NGR234 convey the host range extension and its mobilization into heterologous rhizobia made trans-conjugants which could nodulate non host legumes (Lewin et al., 1990). Another *Rhizobium* sp. LPU83 strain could have extended nodulation host-range and their plasmid pLPU83a can be transferred to different *Rhizobium* species (Gonzalo et al., 2011). Thus the relative permissiveness of the hosts may not guarantee effectiveness in N₂-fixation and may actually lead to the formation of ineffective nodules (Andrade et al., 2002).

Electroporation is also a strategy to introduce a foreign DNA into bacterial genome. Using this technique, Garg et al. (1999) successfully achieved transformation of 15 kb plasmid, pMP154 containing a *nodABC-lacZ* into *Rhizobium leguminosarum* for the improvement of nitrogen fixation. Louise et al. (2002) developed three transposon induced mutants of ANU794 (W72, W78 and W710) which significantly enhanced symbiosis with *Woogenellup*. Musiyiwa et al. (2005) reported genetically modified *Rhizobium* which showed high level of effectiveness in terms of symbiotic ratio for two different cultivars. Ikbali et al. (2017) developed promiscuous rhizobia through conjugation between *Mesorhizobium* and *Rhizobium leguminosarum* and found the transconjugants with better characteristics in nodulation and nitrogen fixation for diverse rabi legumes. Similarly, Yoshitake et al. (2010) genetically modified *Rhizobium* for high catalase enzyme activity. The modified strains were inoculated to the host plant (*Phaseolus vulgaris* L.) and observed the improvement in nitrogen fixation. *Rhizobium* can also exchange plasmid naturally even in the drastic conditions which could be effective for their symbiotic properties. Quorum sensing is one of the more common mechanisms involves in naturally conjugative transfer (CT) described in rhizobia (Lucas et al., 2019). Other molecules, such as flavonoids, carbon compounds or those present in plant exudates, could be involved in the activation of CT. Furthermore, environmental conditions such as pH, nutrient availability, temperature, herbicides and moisture have a significant effect on legume-*Rhizobium* symbiosis and its nitrogen fixation efficiency (Zorawar and Guriqbal, 2018). Defez et al. (2000) successfully transferred 10 kb DNA fragment from *Sinorhizobium* (wild type strain) to *Rhizobium etlii* which was a sensitive strain. The transferred rhizobia effectively nodulated Acacia at 4% NaCl, low and high pH, even in presence of heavy metal and high temperature (43 °C). Similar reports are published earlier on improvement of symbiotic properties of diverse group of rhizobia (Hubbell et al., 1978; Martinez-Molina et al., 1979; Angle, 1986). A predominant and symbiotically effective isolate of this type could prove to be more competitive (Kundu and Dudeja, 2008; Nadwani and Dudeja 2013). The variability in nodulation and nitrogen fixation suggested that the dominant genotype might be the most efficient one for a particular host. The availability of effective multilegume biofertilizer could

avoid the problem of competitiveness. Application of such types of inoculation may avert the use of chemical fertilizers (Ambreen et al., 2012).

3.9 Conclusion

Nitrogen applied as fertilizers usually provides high yields to plants. Therefore, efficient monitoring of plant nitrogen status and appropriate nitrogen fertilizer management are essential to equilibrate crop yield and minimize environmental perturbation, especially water and soil quality. In recent year's considerable efforts has been made to understand the effectiveness of endosymbiosis and microbes host regulation. The inferences from these investigations have enabled us to value add by a microbial inoculant in the form of biofertilizer and also provide basis for further research on diverse *Rhizobium* species. Some genetic determinants of rhizobia involved in host range infectivity have been worked out leading to the extension of their infectivity and effectivity in terms of nitrogen fixation in enlarged host. Thus remarkable research efforts have been carried out for improving the efficiency of biological nitrogen fixation because this process has the potential to reduce our dependence on nitrogenous chemical fertilizers. Multilegume biofertilizer proved better inoculants to improve growth and nitrogen fixation in agricultural practices.

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Chapter 4

The effect of incompatible plant pathogens on the host plant

Laith Khalil Tawfeeq Al-Ani^{1,2} and Edson Luiz Furtado³

¹Department of plant protection, College of Agriculture, University of Baghdad, Baghdad, Iraq, ²School of Biology Science, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia, ³Department of Plant Production, College of Agronomic Science Fazenda Experimental Lageado, São Paulo State University, São Paulo, Brazil

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4.1 Introduction

Plant pathogens are forming a huge problem on the economic and life stability. The plant pathogens are increasing in the wide world. The plant pathogens comprise viruses, bacteria, fungi, nematode, and parasitic plant. The plant pathogens cause the diseases for leaf, stem, root, vascular system and fruit. The plant pathogen attacks the plant by using some mechanisms that are responsible for increasing the disease and appearance the symptoms. The success in the interaction between plant and plant pathogens is causing a full infection that called a compatible interaction. The mechanisms of virulence include (1) a composition of phenotype and genetic, (2) the life history, (3) mode(s) of transmission and (4) environmental factors (Nishiguchi et al., 2008). While another case a plant pathogen can attack the plants and growth inside the plant without causing the infection that called incompatibility interaction behavior the endophyte microbe system. Therefore, many genera of different plant pathogens invade the surface plant but not all these plant pathogens can cause the plant disease.

However, the obtaining of a successful infection is need to several factors. These factors include (A) surface receptors. (B) Produce enzymes, secondary metabolites, and toxins. (C) The capability of plant pathogen for reaching the full infection occurs after overcomes on the plant defenses. (D) The nature of plant includes biochemical, genetic, and physiology of plant. Any defect in the pathogenicity factors leads to losses in the ability of the plant pathogens for causing the infection. Therefore, the appearing nonpathogenic or avirulence of plant pathogens can exist around the plant in rhizosphere and phyllosphere, but some plant pathogens live inside a plant as the endophyte.

Avirulent of plant pathogens might be under three cases (1) Avirulent cannot colonize the plant, (2) Avirulent elicit the hypersensitivity reaction (HR), (3) Virulent harbor the Avr gene (Surico, 2013). Many elicitors and effectors were produced through invading the host plant by an incompatible strain of plant pathogen. Yu et al. (2016) mention for the role of incompatible pathogen strain *Trichothecium roseum* was producing many elicitors that induced the content of total phenolic and flavonoid, lignin deposition, and defense enzymes. In this case, the fungal elicitors was recognizing by plant and led to stimulate the plant immunity in host plant leading to prevent the permeation of incompatible strain inside of the host plant (Kushalappa et al., 2016). The incompatible pathogen strain secreted the effectors (Proteins responsible on suppressing the plant defence of the plant) inside the plant but plant was not recognized. Therefore, the incompatible pathogen strain could not invaders the plant (Selin et al., 2016). The genome of plant pathogen may different between avirulent strain and virulent strain. As example, the avirulent strain of plant pathogen *Serratia marcescens* differed in genomic from the virulent strain about ranging 200–700 bp (Zhang et al., 2005).

However, the incompatibility and avirulent isolate of plant pathogens play an interesting role in the field. There are found many plant pathogens as avirulent for the host or non-host that includes nonpathogenic and incompatible strains. For nonpathogenic strains, are more existing in species of plant pathogens such as plant fungal pathogen such as *Fusarium* (Alabouvette and Olivain, 2002; Al-Ani, 2017b), *Rhizoctonia* (Simonetta et al., 2007), *Colletotrichum* (Askew et al., 2009; Ishikawa et al., 2012), and *Phytophthora nicotianae* (Able et al., 2000). Plant virus pathogen such as cucumber mosaic virus (Karasawa et al., 1999; Shi et al., 2002; Kang et al., 2005). Plant bacteria pathogen *Pseudomonas* (Mohr et al., 2008). Plant parasitic nematodes such as *Meloidogyne incognita* (McKenry and Anwar, 2007). Several avirulent strains and natural product are used controlling of plant pathogens, as well as, the pests and (Al-Ani, 2006; Al-Ani and Salleh, 2010; Mohammed et al., 2011, 2012, 2013, 2014; Al-Ani and Al-Ani, 2011; Al-Ani et al., 2012; Al-Ani et al., 2013; Al-Ani 2017a,b,c; Al-Ani and Albaayit, 2018a,b; Al-Ani et al., 2018; Al-Ani, 2018a,b; Al-Ani, 2019a, b,c,d,e,f). The avirulent strain could grow on the plant surface and in the external cells as epidermal or in the spaces among cells of the host plant (Simonetta et al., 2007). The prevalence of different virulent strains of plant pathogens is leading to happen the mixing infection among these strains on the host or non-host may be leading to appear a new strain located between avirulence to high virulence. The plant host has an influence between negative to positive on the genes of the plant pathogen causing a change in the ability of virulence. It can divide the avirulent strains into four kinds; (A) virulent isolate is an interaction with the non-host plant but it cannot cause the infection, (B) The interaction of avirulent strain with a non-host plant. (C) The interaction of avirulent strain with a host plant (Being a biocontrol agent). (D) Saprophyte is a plant pathogens but it lost the ability to the parasitism and cannot be a biocontrol agent.

There are some factors affecting on the ability of plant pathogens to cause the infection. These factors can be assumed through determining the conditions both of inside or outside of plant that possibly responsible for reducing the ability of plant pathogens. The factors include, (A) environmental conditions, (B) viral infection for the plant pathogens, (C) latent infection in the plants, (D) kind the host cultivar, and (E) plant species. The role of factors is very interesting that they lead to the occurrence of the plant disease epidemic in the fields. It might the three relationships that happened among the strains of plant pathogens, (A) Competition, (B) Cooperation, (C) Coexistence (Abdullah et al., 2017). The interaction among strains of plant pathogens as particular plant viruses leads to an antagonistic infection or a possible synergism (Superinfection) (Syller and Grupa, 2015). Therefore, the avirulent isolates can divide into two divisions according the relationship with other virulent strain, (1) The Antagonism, (2) The synergism.

4.2 The antagonism relationship of the avirulent strain with virulent strains of the plant pathogens

The plants are surrounded with many of avirulent strains that introduce a huge benefit for the plants by confronting with virulent strains of plant pathogens. The avirulent strain or called nonpathogenic strain cannot cause the infection because they lost the ability to colonize and secrete of the secondary compounds with enzymes affecting on the host cells. These types of strains lose the pathogenicity capability for the host or nonhost plant. Indeed, many interactions may happen between the nonpathogenic strain and virulent strain on the host plant which is utilizing for controlling the virulent strain of plant pathogens. Therefore, the competition almost happened between two or more of strains of plant pathogen to create physical barriers and producing the toxins (Abdullah et al., 2017). The antagonism of avirulent strain or nonpathogenic strain is more interesting for controlling the plant pathogens. Many nonpathogenic strains were shown high efficacy against the virulent strains. As particular, it is using same species of plant pathogens as example non-pathogenic *Fusarium oxysporum* to control virulent strain *F. oxysporum*. The efficacy of nonpathogenic strains can determine within some important mechanisms and very useful against a virulent strain of plant pathogens. The ability of nonpathogenic strains in controlling of plant pathogens can determine through some mechanisms including (A) competition, (B) Mycoparasitism, (C) produce the enzymes, antibiosis, and secondary compounds, (D) Induce the defense and resistance of the plant, (E) Plant immunity, (F) Affect on the plant chemistry, (G) detoxification, (H) Hypovirulence, and (K) Cross-Protection. Al-Ani (2019) was mention for several mechanisms of nonpathogen plant pathogen. The success of controlling the virulent strains was related to two factors, (A) the efficacy of mechanisms of nonpathogenic strain and (B) Host, the plant species (Alabouvette and Olivain, 2002).

Surprisingly, for the competition, the nonpathogenic strain showed very high competition with other virulent strains. The nonpathogenic *F. oxysporum* Fo47 strain found to reduce the activity of metabolites and the population density of the virulent strain of plant pathogens *F. oxysporum* f. sp. *lini* on the root (Duijff et al., 1999). For the mycoparasitism, endophytic *Fusarium* spp. was able to parasitism on the asexual structure of *Sclerotinia sclerotiorum* (Tsapikounis, 2015). Nonpathogenic strain both of Oomycota *Pythium oligandrum* and *Pyth. periplocum* were able to parasitism on

other fungi such as *Verticillium dahliae*, *Botrytis cinerea*, and dermatophytes fungi (Al-Rawahi and Hancock, 1998; Paul and Masih, 2000; Gabrielová et al., 2018).

For the enzymes, antibiosis, and secondary compounds, many hydrolytic enzymes and antibiotics produced by the endophytic *F. proliferatum* strain I92 and affecting fusarium disease in tomato (Nefzi et al., 2018). Nonpathogenic *Fusarium oxysporum* strain CanR-46 produced many volatile compounds having the ability to inhibit the plant pathogen *Verticillium dahliae* (Zhang et al., 2015). Several strains of nonpathogenic *Fusarium* affected on plant pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (FocTR4) by producing siderophores (Al-Ani, 2017b).

For the induce of plant defence, was elucidated through improving (A) the contents of total protein and phenol, (B) polyphenol oxidase, and (C) activities of peroxidase in tomato after challenging with Fu3 strain of nonpathogenic *Fusarium moniliforme* against Fusarium wilt (Patil et al., 2011). The change in plant physiology such as accumulation of phenol compounds, callus around the penetration place, nuclear migration, rapid cytoplasm aggregation, superoxide release, and hypersensitive cell death was a response of plant defense against plant pathogens that elicited by the inducer factor (Daniel and Guest, 2006). The changes in the activity of the enzyme such as chitinase and peroxidase in the host plant after treating with the inducer were considered as an indicator of the occurrence of resistance (Boava et al., 2010). Thus, the plant resistance was shown in some plant after treating with nonpathogenic. Reuveni and Reuveni (2000) found two isolates of nonpathogenic fungi such as *Alternaria cucumarina* or *Cladosporium fulvum* could induce resistance in cucumber against a plant pathogen of powdery mildew *Sphaerotheca fuliginea* after pre-inoculation with them. The Fo47 strain induced three genes such as PR-1a, CHI3, and GLUA in the tomato root by pre-inoculation treatment (Aimé et al., 2013, 2008). While, plant immunity, was elucidated a plant pathogen-induced gene silencing-based on the secretion of the pathogen-associated molecular pattern that stimulated signaling pathways of the immunity in the plant against a virulent strain of plant pathogen (Segonzac et al., 2011). This stimulated leading to systemic acquired resistance and priming (Ramirez-Prado et al., 2018). PAMP-triggered immunity caused growth inhibition and restricted the proliferation of plant pathogen (Zhang and Zhou, 2010; Segonzac et al., 2011).

In additional, avirulent strains of the plant pathogen *Xanthomonas campestris* induced the change in the volatile compounds of plant (Ponzio et al., 2016). On the other hand, many fungal and plant pathogens produced very harmful mycotoxins (Attitalla et al., 2010a,b). Fakhouri et al. (2003) detected the role of nonpathogenic *Colletotrichum* sp. in detoxifying the mycotoxin fusaric acid and converted it into 4-butyl-2-carboxy-pyrimidine. However, hypovirulence of a virulent strain means a reduction in the ability of a strain to infect the host plant. This decrease in the pathogenicity become from mixing the virulent strain with an avirulent strain (Already Infected with mycovirus) to transfer the mycovirus. Pereira et al. (2015) using the hypovirulent strain of *Cryphonectria parasitica* (Infected with *Cryphonectria hypovirus* 1 (dsRNA CHV1)) against the virulent strain of *C. parasitica* was decreased the influence of the virulent strain and also it enhanced the plant healthy.

In addition, cross-protection method of biological control can elucidate by treating the plant with a mild or weak strain of plant pathogens as induce the pre-immunity or increase the tolerance against a virulent strain of plant pathogens. The mild or weak strain is the ability to stimulate the defence and resistance of plant. The cross-protection of utilizing the mild plant strain virus against a virulent strain called an acquired immunity phenomenon (Zhou and Zhou, 2012). They found happen several changes such as the pathways of signaling, enhancing the cell wall, energizing of hormone metabolism, secondary metabolites, and in the host plant as response defense to treat with some strains of plant pathogen (Fu et al., 2017). This method of control can divide into two subdivisions, as following:

1. Mild or weak strain against virulent strain of same group plant pathogens such as virus against virus, fungi against fungi, etc. They were decreased the effect virulent strain of plant virus Citrus Tristeza virus by treating with a mild strain of Citrus Tristeza virus (Balaraman and Ramakrishnan, 1980; Folimonova, 2013; Leonel et al., 2015).
2. Plant pathogen against another plant pathogen such as virus against fungi, or vice versa, etc. They detected of ability plant pathogen virus Zucchini yellow mosaic virus (ZYMV) induce the systemic acquired resistance (SAR) in zucchini plant by increasing in levels of phytohormone as particular salicylic acid against another plant pathogens powder mildew (Harth et al., 2018).

4.3 The synergism relationship of the avirulent strain with virulent strains of the plant pathogens

Many populations of plant pathogens are attacking the plants but some strains of the plant pathogens don't have the ability to cause the infection. The strains of plant pathogen failed to cause the infection can call incompatible interaction. Also, Chappell and Rausher (2016) mention for the virulent strain of the pathogen genotypes was a specificity

pathogen for one host in a native of plant community but in a non-native of plant community could infect broad hosts not specific for one host. Therefore, the incompatible strain in local of plant community such as place (A) may be compatible strain in another plant community such as place (B). However, the fungal hyphae of an incompatible strain of plant pathogen *Tilletia tritici* were surrounded with more layers of callose in the infected tissue of wheat as result from infection with a compatible strain of *T. tritici* (Gaudet et al., 2007). The accumulation of callose around the fungal hyphae is causing a restriction of nutrients to reach to fungal hyphae then stops the colonization and inhibits the fungal growth. The cooperation between highly virulent strains with incompatible strain was occurrence by sharing in the essential biochemical signals for the pathogenesis or exchanges the nutrients useful for the survival (Abdullah et al., 2017). The incompatible strain of plant pathogen *Pseudomonas syringae* increased in the growth on the nonhost through existing with the virulent strain *P. syringae* (Barrett et al., 2011). Zhang et al. (2001) mention to importance determined the role of interaction among strains of the plant pathogen having the epidemiological implications in the fields. This might be incompatible strain lost some important traits that require causing the pathogenicity such as:

- (A) Production the secondary metabolites such as phytotoxins, and other compounds such as fusaric acid. The restriction of mycotoxins production is affecting on the virulence of plant fungal pathogens (Wu et al., 2009).
- (B) The important enzymes production is responsible for the degradation of the tissue and cell walls such as a cutinase. The efficacy of strains from incompatible strain to high virulent strain was depended on the ability to secrete the cell wall degrading enzymes such as pectinesterase, pectinase, and cellulase, as well as, cutinase (Köller et al., 1982). Many enzymes of cell wall-degrading enzymes such as pectin lyase, pectatylase, polymethyl-galacturonase, polygalacturonase, β -galactosidase and cellulase, were produced by the virulent strain of plant pathogen (Zhang et al., 2014; Xue et al., 2018).
- (C) The ability to confront the plant defense. The virulent strain has the ability to confront the plant defence of the host by secreting some secondary compounds and enzymes (Hok et al., 2010), such as toxins and cell wall-degrading enzymes.
- (D) Definitely the gene compatibility according to the pathogen-plant interaction as the gene to gene (Staskawicz, 2001). The role of a gene to gene was important for the interaction between the virulent strain and the host plant to suppress the plant defence and appear the disease on the host plant (Sonah et al., 2016).
- (E) Other factors such as environmental (micro and macro environmental) and biological (Biological control agents) prevents the compatible interaction between the strain of plant pathogens and host.
- (F) A strain of plant pathogens attacks non-host leading to the incompatible between them. The incompatible strain of plant pathogens is possible growth on the surface of the host without causing the infection. The existence of the plant pathogen *Fusarium graminearum* on some cereal weeds without caused any infection as incompatible strain but it was a virulent strain for the cereals (Suproniene et al., 2019).
- (G) A strain of plant pathogens has a virus infection (infected with dsRNA). The mycovirus played a role in stopping expression for the gene of pathogenicity enzyme as particular Cutinase that modify in the virulent ability of plant fungal pathogen to be a hypovirulent strain (Varley et al., 1992). The purpose of listing these factors is to begin to clarify the specific idea of the role of avirulent strains as a result of incompatibility in the spread of plant disease epidemics through collaboration with a virulent strain of plant pathogens.
- (H) In an incompatible interaction (host resistant) there is an early but localized increase in chalcone synthase mRNA activity prior to the onset of accumulation of the phenylpropanoid-derived phytoalexin phaseollin and expression of hypersensitive resistance. In contrast, in a compatible interaction (host susceptible) there is no induction of mRNA activity in the early stages of infection but rather delayed, widespread increase during attempted lesion limitation at the onset of symptom development. The data indicate that of control phytoalexin gene expression is a key early component in the defense responses of biologically stressed cells during a race-cultivar specific host pathogen interaction (Bell et al., 1984).

However, this incompatible strain of plant pathogens that attack host or non-host may exist in phyllosphere and endophyte as assume remain. The existence of incompatible strain is forming a side effect on the host. This strain in original is from the plant pathogen; therefore, it almost still forms the dangerous on the host or in the fields. The harmful role of incompatible strain is showing through a presence the virulent strain of plant pathogen can possibly happen a synergism as assume. The incompatible strain of plant pathogen *Pseudomonas syringae* 503 was unable to cause the infection for three host plants such as *Nicotiana benthamiana*, *Nic. tabacum*, and *Arabidopsis thaliana*, because of the level of population untypical to cause the disease (Mohr et al., 2008). Al-Ani (2017b) isolated some incompatible strains of plant fungal pathogen including two isolates of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (FocTR4) and one isolate of endophyte *F. proliferatum*. These isolates showed the dangerous role by synergistic with a virulent

strain *FocTR4* to cause the fusarium wilt for banana and high damage for the banana tissue compared the infection with a virulent strain alone (Data unprinted).

Interestingly, this experiment was indicated for the potential happen the cooperation between strains of a plant pathogen. This result reveals cooperation among strains of plant fungal pathogen either same species or different species. The theory of cooperation between incompatible strain (Avirulent) and compatible strain (Virulent) is acceptable because the tissue of banana that infected with the incompatible strain and compatible strain was been more damaged compared with the tissue banana infected with compatible strain only. There are many assumptions about the cooperation can mention here. The cooperation between two strains is useful in the two sides; first side for incompatible strain and second side for virulent strain. In the first side, the growth, activity, proliferation, and survive of incompatible are very limited and virulent strain can provide the suitable conditions for the incompatible strain.

In additional, the incompatible strain cannot grow and colonize the tissue of banana due to unavailable the unsuitable factors. The virulent strain is having the ability to affect some unsuitable factors and change them to become more suitable conditions for the incompatible strain. Thus, the suitable conditions may become useful for the incompatible strain to complete the typical life cycle including;

- I) Virulent strain is having the ability in reduction of the defence and resistance capability of the host plant. The virulent strain produced several types of pathogen factors including proteins, peptides, enzymes, and secondary compounds such as toxins that affected the defenses of the host (Wirthmueller et al., 2013; Giraldo and Valent, 2013; Stergiopoulos et al., 2013). They found that the ability of virulent strain for a plant pathogen to infect on NO that responsible on the plant defense responses (Zeier et al., 2004). The virulent strain was produced the nepi-like protein (NLPs) such as toxins causing a cytolytic for the host plant tissues (Küfner et al., 2009). The existence of avirulent strain evidenced for the theory of gene-to-gene and unable to secrete the effector proteins a responsible to cause the infection for the host plant (De Wit et al., 2009). The pathogenicity factor such as the effector proteins that secreted from the virulent strain was responsible on destructing the cell tissues with affecting on the plant defense for the host plant (Sonah et al., 2016). The suppression of accumulation the phenols in the cell wall and the inhibition of phenylalanine ammonia lyase (PAL) were obtained through the activity of virulent strain (Aoun, 2017). The suppression of plant immunity enhanced the host infection by a virulent strain *Phytophthora infestans* happened due to the role Pi02860 (Effector) of stimulation the association between SWAP70q (Guanine nucleotide) and NRL1 (Host protein) to encourage the proteasome-mediated degradation then reduce in plant immunity (He et al., 2018). Interestingly, this status is the suitable condition to decrease the defense ability of the host and colonization by incompatible strain. The role of incompatible strain is supporting behind the virulent strain by secreting the enzymes and secondary compounds such as toxins.
- II) The virulent strain impact the primary metabolites such as photosynthesis and growth leading to the general weakness in the plant body. Because it is weak in the cell wall component such as the starch, carbohydrate, and others that lost through decreasing in the photosynthesis activity or full suppressing as result from the secondary compounds or enzymes secreting by the plant pathogens. Berger et al. (2007) mention the ability of plant pathogens in reducing the primary metabolites such as growth, development, and photosynthesis. Finally, the general weakness in the plant structure enables the incompatible strain to invade the plant and colonize.
- III) Secreting some enzymes by virulent strain for degrading the cell walls of the host tissues that provides the useful nutrients for the typical life cycle. In return, these nutrients may induce the incompatible strain to produce the toxins, enzymes, and secondary relevant that using in the pathogenicity. They detected the role of some enzymes such as cutinase that degraded the plant cuticle and then colonized the host plant (Varley et al., 1992). Many substrates produced after a degradation of cell wall. Therefore, the degradation of the cell wall facilitates for the incompatible strain to colonize the tissues of plant host and it may stimulate to produce the effector protein and toxins impacting on the host cells. Liu et al. (2016) detected the important role of some substrate such as sucrose, glucose, arginine, glutamic acid, aspartic acid, Maltose, and zinc in the production of mycotoxins. Virulent strains produced the cutinase enzyme to degrade the cutin in the cell wall as a carbon source for growth (Dickman and Patil, 1986). In additional, cutinase enzyme could be an indicator of the type of plant pathogen strain either virulent or avirulent (Fan and Köller, 1998; Lu et al., 2018).
- IV) Virulent strains are possible producing some enzymes and secondary compounds appropriately for removing the inhibitory compounds existence in the habitat that secreted by the useful microorganisms such as PGPR and *Trichoderma*, etc.
- V) Production of some antimicrobial materials restrict are working on the restriction of the useful microbe growth, this provides a suitable chance to incompatible strain for the growth and completes the typical life cycle on the

host appearing the disease symptoms. The virulence strain produced the siderophores as an instrument for competition with the host and other microorganisms (Aznar and Dellagi, 2015), that may affect the growth of useful organisms in the niche.

- VI) The co-existence of two strains of same species may happen a parasexual recombination between incompatible strain and virulent strain leading to appear a new strain with a new trait and may be very virulent. Ishikawa et al. (2012) detected the possible occurrence the horizontal gene transfer in vegetative phase between two strain of plant pathogen *Colletotrichum lindemuthianum* such as incompatible and compatible strain that leading to appear a new virulent of this plant pathogen.
- VII) Virulent strains can produce some enzymes and secondary compounds inducing the incompatible strain for growing and complete the typical life cycle.
- VIII) Virulent strains are useful for the weak strain by helping the multiplication in the tissues of the host plant. This phenomenon can happen through mixing infection among strain of plant pathogens. The co-infection between two or more than two strains of plant pathogens affects on (A) diseases severity, (B) transmission such as for plant virus, (C) Proliferation or multiplication, and (D) occurrence the epidemic for the rate of the infection spread in the field. The synergism among plant virus strains may be leading to occur the epidemiology for the plant virus infection (Zhang et al., 2001). As an example, Pio-Ribeiro et al. (1978) found the severe symptoms on cowpea cultivars as resulted from the synergism between Cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BICMV). The co-infection between CMV and BICMV affected the plant physiology of cowpea plant that showed symptoms of very severe (Anthony, 2019). The interaction among virulent strain of plant virus included Sweet potato feathery mottle virus (SPFMV), and a mild strain of plant virus. Sweet potato chlorotic stunt virus (SPCSV) caused very severe symptoms such as severe symptoms in leaves and stunting of the potato sweet (Karyeija et al., 2000). The occurrence of synergism between the Potyvirus genus CMV could happen in the infecting of the cucurbits (Wang et al., 2002; Barbosa et al., 2016). That means the increase in infection and disease severity with a difference in symptoms compared without synergism between them. The mix infection between CMV and ZYMV caused abnormal distortions on cucumber compared infection with CMV only showing oil spot symptom on cucumber (Lobin et al., 2015). They mixed infection among three unrelated viruses including Southern bean mosaic virus (SBMV), Cowpea aphid-borne mosaic virus (CABMV), and Cowpea mottle virus (CMeV) on several cowpea cultivars causing severe symptoms compared with single infection of these viruses on cowpea cultivars (Nsa and Kareem, 2015). While Arogundade et al. (2009) mention to a little or no synergistic happened between CMV and CMeV on the soybean cultivars. This indicates the role of plant host in the occurrence of synergism among viruses.

A second side, the virulent strain is confronting some unsuitable factors possible are leading to reduce the capability to cause a typical infection for the host. The incompatible strain may speed up in a role of virulent strain in dominance on the host tissues. The speedup in the colonization of the virulent strain for the host tissues is meaning the change in optimum of the life cycle and formation of the dispersal stage that is responsible for spreading the secondary infection to other plants in the field. The establishment of secondary infection (as of example spores or other depending on kind of plant pathogens) leads to an increase in spreading of infection in the field. This method of infection is significant loss on the crops and vegetables that may be leading to occur the epidemic for this disease. The incompatible strain is having the ability to protect the cell walls for the virulent strain from effects of the plant defenses. They found the incompatible strain of plant pathogen *Cladosporium fulvum* playing a role in protection the chitin of fungal cells *Fusarium solani* f. sp. phaseoli from hydrolysis by the chitinases that a host plant secreted it (van den Burg et al., 2006).

4.4 Conclusion

In the fields of crop, vegetables, and other types of plants, are living many strains of plant pathogens between avirulent to high virulent strains. This difference of population leads to two interactions being useful for the plant in some status but the opposite side form a significant danger on the host plant may lead to the occurrence of the plant disease epidemic in the fields. The useful interaction between the nonpathogenic strain and virulent strain for the host plant is appearing in reduction of the symptoms of a plant disease in the fields.

Interestingly, the activity of nonpathogenic is keeping in the high potential in affecting the able of virulent strain to cause the infection. Nonpathogenic strains have several effect mechanisms showing efficacy in reducing the activity of plant pathogens both of direct and indirect action. Two mechanisms of nonpathogenic strains such as competition and

their production of enzymes and secondary metabolites are showing more effect on the activity of virulent strains of plant pathogens. On the other hand, the harmful side is clear in the prevalence of incompatible strain in the fields. The incompatible strain of plant pathogen cannot cause the infection under in some unsuitable conditions but it is having the ability to cooperate with a virulent strain for the destruction of the host plant. However, it can utilize the hypovirulence strain to control of virulent fungal strain by transferring the infection of mycoviral to another virulent strain. As well, the possible cross-protection utilize the weak or mild strain against another virulent strain, and plant pathogen against another plant pathogen showing the efficacy to control the virulent strain through the ability to induce the plant immunity against another virulent strain or plant pathogen

However, the cooperation between incompatible strains with avirulent strain is obtaining through attacking and colonizes the tissue of the host plant. In this case, the virulent strain is possible play a role in introducing a service for the incompatible strain by providing suitable conditions. We assumed eight points (see Section 4.3) about the ability of the virulent strain to helpful the incompatible strain like as breakage the layers of plant defense and providing the important elements. These steps are to encourage of incompatible strain to growth and production the enzymes with secondary compounds that more necessary for colonizing the host cells without the need to get compatible with the host plant (The gene to gene). While there is evidences to potential the incompatible strain exhibiting some services for a virulent strain of plant pathogen. The role of incompatible strain in cooperation with virulent strain to colonize the host tissues of plant is contributing in the fast dispersal of plant pathogens and prominence the secondary infection quickly. The fast occurrence of secondary infection for the plant pathogens in the fields leads to dispersal the infection through the short period compared with the standard period for the optimal occurrence of infection in the normal conditions. This condition is suitable for getting a plant disease epidemic in the field that may be spread in a big area and causes the severe losses in the form of economic crops and vegetables, as well as, orchards. The synergism between the strains of plant pathogens is leading to occur more disease severity and may cause the epidemic in the fields because it happened the change in the rate of disease spread.

Finally, the blending among strains of plant pathogens may lead to obtain the plant disease epidemic. The mixing among plant pathogens strains considers to include a group of effective factors that possible cause the plant disease epidemic. Therefore, it must find some restrictions through utilizing the avirulent strain (as biological control agent) to control of plant pathogens but it must be in the restriction places such as a plastic or glassy house. This suggestion is to prevent dispersal the virulent strain in the fields and occurrence the mixing with other strain of plant pathogens. Using nonpathogenic strain as a biological control agent is the best step in using against a virulent strain to potential a similarity in the site of infection through colonizing the host plant. Some nonpathogenic strain have a high efficiency in control of the plant pathogens to competition on the infection site without affecting on the other host plant to similarity in the species such as nonpathogenic *Fusarium oxysporum* Fo47. Therefore, a nonpathogenic strain may be useful to control of plant disease without using the chemical pesticides but it must select the nonpathogenic strain within limited criteria to prevent the creation of a new virulent strain of plant pathogen. However, as the assumption in the occurrence of plant disease epidemic as a result of some plant pathogens, as particular *Fusarium oxysporum*, *Rhizoctonia solani*, and *Colletotrichum* spp. may get from the accumulations of many avirulent strains and interaction with a virulent strain in the fields. This interaction is parasexual recombination. As well, the change and stability in a genes nature for the strain of plant pathogen are difficult but after a long time of interactions among the blend of plant pathogen strains, it is leading to prominence a new virulent strain that is more destructive for the plant.

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Chapter 5

Language of plant-microbe-microbe interactions in rhizospheric ecosystems

Ekta Khare¹, Swati Tyagi² and Kishor Sureshbhai Patil³

¹Department of Microbiology, Chhatrapati Shahu Ji Maharaj University, Kanpur, India, ²Genomics Division, National Academy of Agriculture Science, Rural Development Administration, Jeonju, Republic of Korea, ³Division of Biotechnology, Chonbuk National University, Iksan, Republic of Korea

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5.1 Introduction

Plant roots form a dynamic front or region named as rhizosphere. It is a nutrient rich environment that promotes complex microbial communities, and where plant roots and beneficial/ pathogenic microbes interact for each other's benefit (Tyagi et al., 2018). The term 'rhizosphere' was coined by the German scientist Hiltner in 1904 for that region of the soil which is subject to the affect of plant roots and assumed to extend from the root surface into the soil to a few millimeters (Hiltner, 1904). Rhizosphere can be parted into – (a) the rhizoplane (i.e., the surface of root), (b) the endorhizosphere (i.e., the internal root parts) and (c) the ectorhizosphere (the thin layer of sand or soil adhering to roots). Microbial density in the rhizosphere is 10–1000 times greater than that in the bulk soil (Hiltner, 1904). In the rhizosphere, a biologically and chemically diverse, intricate and dynamic interaction happens between plant roots, soil (micro) biota and the physiochemical state of the soil.

Studies conducted recently have showed that plant species choose a particular rhizosphere bacterial community (Lareen et al., 2016; Ho et al., 2017). The microbial community (may harbor harmful or beneficial members) in the rhizosphere gives it's input on the plant relative performance that clarifies the long-term consequences of the soil microbes on their togetherness with that plant species (Ho et al., 2017). Composition of rhizosphere microbial communities can effect phyto-pathogen growth, nutrient availability and plant health (Srivastava, 2015). Within the rhizosphere microbial populations, rhizobacteria can be classified among beneficial, deleterious, and neutral groups on the foundation of their effects on plant's development. Neutral group of bacteria does not have a particular effect on plant's health, but they are a segment of an intricate food web which uses the huge amount of carbon fixed by plants that released into the rhizosphere (i.e. rhizodeposits). Beneficial rhizobacteria that promotes plant growth either directly or indirectly are generally referred to as Plant Growth Promoting Rhizobacteria or PGPR (Glick, 1995). On the other hand, deleterious rhizobacteria are the minor pathogens that produce phyto-toxins, enzymes or hormones, which can be in a competition with PGPR for nutrients and effects health of plant without parasitizing plant tissues (Nehl et al., 1997).

An accomplishment of a particular effect by a microbial community on to the host largely depends on its potential to perceive, process and quick response to transformations in the local environment (pH, temperature, moisture, nutrient availability, microbial population etc.). For a specific bacterium, one of the biggest challenges is the number and

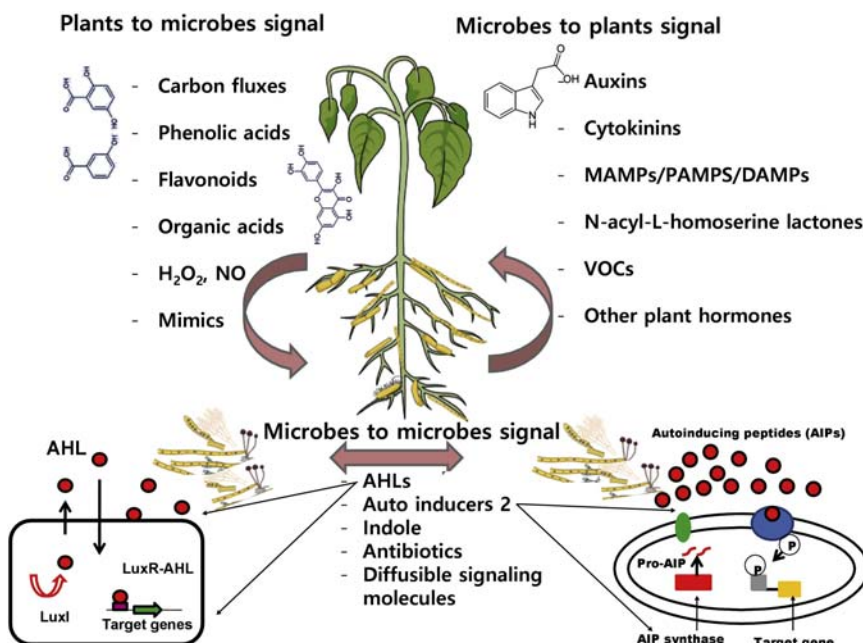


FIGURE 5.1 Summary of signal molecules governing plant-microbe-microbe interactions.

development of its fellow in its near proximity. Such knowledge might permit the bacterium to expect nutrient availability or need of toxins. It permits bacterium to coordinate and create new ways to survive, that won't be feasible by one's own acts. The capacity to look at the local population density depends on a cell-to-cell communication system called quorum sensing (QS) (Fray, 2002).

To identify, respond or defend against microorganisms (pathogenic and beneficial), plants have developed a complex immune system that "perceive" signals from microorganisms and process, integrate or translate that "perception" into a suitable adaptive response (Pozo et al., 2005; Pineda et al., 2013). Different forces structuring plant communities, plant-microbe interactions have got very little attention (Thompson et al., 2001). In this book chapter, what is to be considered by us is the plant-microbe-microbe interactions with regards to the dynamic changes in the signaling network, and multidirectional comprehension of these communications (Fig. 5.1). Study of these co-operative microbial interactions can be exploited as a low-input biotechnology, and it can create the foundation for the strategy to help sustainable, environmental-friendly ways which are basic to the stability and productivity of agricultural systems and natural ecosystems.

5.2 Plants to microbes signals

At root-soil interface, microorganisms interact with roots and rhizospheric constituents which results in the development of dynamic carbon rich environment (Lynch, 1990; Kennedy, 1998; Barea et al., 2002). The source of carbon compounds for the soil biota comes from the plant root exudates and decaying plant material, serve as either growth substrates or signal molecules (Degenhardt et al., 2003). These compounds can be classified into low-molecular weight, high-molecular weight and volatile organic compounds (VOCs). Low-molecular weight compounds include sugars, amino acids, organic acids, phenolics, vitamins and various secondary metabolites, are the main component of exudates (Arora and Khare, 2008). High-molecular weight compounds are comprised of mucilage and proteins. The major portion of volatile compounds are carbon dioxide, certain secondary metabolites, alcohols and aldehydes (Schulz and Dickschat, 2007; Badri and Vivanco, 2009; Tyagi et al., 2018).

Carbon fluxes play an important role in rhizosphere functions (Toal et al., 2000). Plant released sugars are known for the effect on growth and development of microorganisms present in their rhizosphere, through the induction in expression of genes required for their uptake and catabolism (Jha and Subramanian, 2018). Glucose and galactose are exuded from roots as apart of plant cell wall polysaccharides. The sugar binding protein ChvE of *Agrobacterium tumefaciens* not only contribute to the chemotaxis of bacteria but also involved in carbon metabolism. The *chvE* gene is part of an operon and it is transcriptionally induced by glucose or galactose (Kemner et al., 1997; Subramoni et al., 2014).

In addition, organic acids and sugars containing root exudates from banana and tomato control biofilm formation and antifungal activity of *Bacillus subtilis* N11 and *Pseudomonas* spp. (Kravchenko et al., 2003; Zhang et al., 2014).

Phenolic acids are chief polyphenols synthesized by plants that are involved in protection against biotic stresses, and are vital in plant-microbe interactions/ symbiosis (Mandal et al., 2010). Phenolic compounds act as signaling molecules in the beginning of legume-rhizobia symbioses, forming arbuscular mycorrhizal symbioses and might play the roles of agents in plant defense. Phenolics such as phytoalexins secreted by wounded or otherwise perturbed plants repel or kill many microorganisms. Few pathogens can counteract or nullify these defenses or even subvert them for their benefit. In soil, phenolics compounds evoke a redox reaction that influences the development of soil microbial community, through the pressure on the hormonal balance, enzymatic activity, availability of phytonutrients and competition between neighboring plants and microorganisms (Hättenschwiler and Vitousek, 2000; Bhattacharya et al., 2010). Flavonoids are a different category of polyphenolic compounds that received a lot of attention as signaling molecules involved in plant-microbe interactions. Flavonoids act as chemo attractants for rhizobia and also induce the expression of nodulation genes (*nod*-genes), hence the synthesis of Nod factor (lipochitooligosaccharide) (Perret et al., 2000; Mandal et al., 2010). Root exudate of legumes contains various flavonoids which exhibit a strong stimulatory effect on the macroconidia germination of the pea and bean pathogen *Fusarium solani* (Ruan et al., 1995).

Several workers reported that the presence of toxic compounds or low availability of phosphate, effects the root exudate composition, particularly the increased secretion of organic acids such as oxalic acid, malic acid and citric acid (Aulakh et al., 2001; Badri et al., 2008). These organic acids/ compounds such as malate might cause microbial attraction, or be used as carbon sources for microbial nutrition. Malic acid secreted from root exudates attract the beneficial soil bacteria to the root and provide protection against the foliar pathogen *Pseudomonas syringae* (Rudrappa et al., 2008). Genetically engineered tobacco and alfalfa plants were created to overproduce citric or malic acid, with an increased colonization by mycorrhizal fungi and rhizobacteria, throws light on the role of organic acids in plant-microbe interactions (Tesfaye et al., 2003).

An extensive range of biotic and abiotic factors stimulate plants for generation of hydrogen peroxide (H_2O_2). It can include pathogen attack, wounds, plant hormones, drought, temperature, radiations and exposure to ozone (Wu et al., 2016; Zhu et al., 2016). H_2O_2 is an attractive signaling molecule that mediates cross signaling pathways, and granting to the phenomenon of 'cross-tolerance'. Few examples of cross-tolerance are the exposure of plants to sub lethal doses of ozone or UV conferred tolerance to infection by a virulent pathogen (Sharma et al., 1996), and exposure to heat stress induced tolerance towards subsequent pathogen attack (Vallelian-Bindschedler et al., 1998). Other than this, H_2O_2 generated due to exposure of plant to low levels of one stress (e.g. cold) can induce tolerance towards succeeding exposure to the higher levels of the same stress, a phenomenon termed 'acclimation tolerance' (Prasad et al., 1994). Nitric oxide (NO) is another stress responsive signal molecule that can be synthesized at the same time as H_2O_2 . In 1998 two milestone researches demonstrated the role of NO during the hypersensitive response (HR) of plants to infection by bacteria and viruses (Delledone et al., 1998; Durner et al., 1998).

The effect of plant species on microbial communities (Borymski et al., 2018) and populations of specific bacterial genera has been documented very well (Lemanceau et al., 1995; Berg et al., 2002; Gu and Mazzola, 2003; Pivato et al., 2017). One of such example is the significant variation in composition of fluorescent *Pseudomonas* spp. within the rhizosphere of different plant species cultivated in the same soil (Lemanceau et al., 1995). The composition of microbial community and soil properties may alter dramatically over time in response to the age-related physiological changes in plant species (Mazzola and Gu, 2000; Arora and Khare, 2008). The relative abundance and functional diversity of microbial groups in soil ecosystems is strongly depend on the type and amount of available organic substrates (Grayston et al., 2001). Fan et al. (1997) analyzed the root exudates of *Hordeum vulgare* and found that the quantities of exudates particularly organic acids and phytosiderophores increase three fold during mild iron stress. Phytosiderophores can be utilized by certain rhizospheric bacteria as a supplier of iron or may alter iron availability to plants and other competing microorganisms (Crowley and Gries, 1994).

Direct or passive diffusion of low molecular weight compounds through the lipid bilayer of the plasma membrane is depends on by membrane permeability. The physiological state of the root cell and the polarity of the compounds determine the membrane permeability, facilitating the permeation of lipophilic exudates (Rudrappan et al., 2007; Nihorimbere et al., 2011). The efficiency of the exudation process may thus be enhanced by various environmental factors affecting membrane integrity such as nutrient deficiency, temperature extremes, soil pH, light intensity, soil type, oxygen status and presence of microorganisms (Singh and Mukerji, 2006; Nihorimbere et al., 2011). The exudation rate and composition determines the functional diversity of an ecosystem. A succession in functional diversity can be observed as initially low in non-vegetated sites, but as vegetation was established, the diversity rapidly increased and, in older regimes, the catabolic evenness declined (Torsvik and Øvreås, 2002).

5.3 Microbes to plant signals

5.3.1 Beneficial interactions

Root exudates releases various carbon compounds into the rhizosphere, increases the population density and activity of microbial community. First move of microorganisms is to find the plant host, followed by colonization in the rhizosphere by stimulating plant growth through production of canonical plant growth-regulating substances such as auxins or cytokinins. Beneficial rhizospheric microorganisms are able to influence plant's development by several mechanisms, such as decomposition and mineralization of organic matter, enhancing the bioavailability of minerals such as phosphorus and iron, production of phytohormones and contribution to plant immunity by production of elicitor molecules or by producing volatile organic compounds (Ortíz-Castro et al., 2009; Tyagi et al., 2018).

5.3.1.1 Nutrients mobilization

Plant developmental process is a complex series of event that is controlled by several internal and external signals that depend on the adequate availability of mineral nutrients in soil. Variations in the intensities of certain nutrients are reported to alter tissue concentrations of cytokinins and abscisic acid, two major signals controlling plant growth hormones (Moorby and Besford, 1983). The most important route by which microbes influence plant productivity, is through enhancement in plant nutrient availability via nutrient mineralization, a process in which soil microbes break down soluble and insoluble organic matter and convert it into inorganic forms (Marcel et al., 2008). Higher microbial activity in the rhizosphere because of the utilization of exudates carbon creates a 'priming effect' that eventually leads the way to increased mineralization of soil organic matter (Cheng, 2009; Chen et al., 2014). However, the more detailed investigations are required for understanding the carbon mineralization coupled with amounts of N and P released (Chen et al., 2014). Whilst C:N ratios are similar across many different types of soils and for various fractions of soil organic matter, however, wider variation in C:P or C: organic P ratios are a general phenomenon (Kirkby et al., 2011).

Proteobacteria are an interesting group of bacteria because of their posited dominance in the rhizosphere. They release of a specific signal molecule known as N-acyl-homoserine lactones, or (AHLs) to make other proteobacteria aware of their presence. This way of sending signals is closely connected with size of bacterial population and AHLs concentration; once threshold concentration of AHLs is achieved, it starts transcription of QS related genes to exhibit cell density dependent phenotypes (Palmer et al., 2014). Other than that, soil bacteria extracellularly release a number of lytic enzymes that produce soluble, digestible molecules into their environment. Higher the number of bacteria, greater the concentration of extracellular enzymes in its surrounding and thus a more nutrient-rich environment for plants (DeAngelis et al., 2008). Dead organic matter or complex insoluble polymers such as proteins, nucleic acids and chitin is the major form of nitrogen in soil. These polymers are further degraded by microbial enzymes into dissolved organic nitrogen and make it easily available to plant and microorganisms (Schimel and Bennett, 2004). Other channels through which microbes can change the plant nutrient availability includes weathering of soils via the exudation of organic acids (Landeweert et al., 2001) and the solubilization of different forms of precipitated phosphorus (P) (Kucey, 1983). Microorganisms also enhance the availability of soluble P to plants via alteration of sorption equilibria. This can be achieved through reduction in soil pH leading to increased net transfer of orthophosphate ions into soil solution, and by microbial turnover of soil organic P (Seeling and Zasoski, 1993). Some rhizospheric microorganisms also produce siderophores; a class of compounds that can chelate the ferric iron from different terrestrial or aquatic habitat and making it available for microbes and plants. These Fe^{3+} -siderophore complexes can be absorbed by many plant species (Bar-Ness et al., 1991; Wang et al., 1993). But still, there is a controversy regarding the part of microbial Fe^{3+} -siderophore complex responsible for the iron nutrition of plants.

A turn-table evidence was provided by Paungfoo-Lonhienne et al. (2010) raising questions on the 'soil microbial loop' concept, that nutrients and carbon are cycled between soil and microbial pools, and the nutrients became available via microbial turnover of soil organic matter and are subsequently 'scavenged' by the plant root (Bonkowski, 2004). Their study with fluorescent-labeled non-pathogenic and non-symbiotic strains of a bacterium (*Escherichia coli*) and a fungus (*Saccharomyces cerevisiae*) reported that microbes enter root cells and are subsequently digested by plant. The nitrogen released in this process was used in shoots, creating a new idea that plants can consume microbes as a source of nutrients (Paungfoo-Lonhienne et al., 2010). Authors further reported that presence of *E. coli* triggers the induction of cellulase expression in *Arabidopsis*, and this may be linked to the uptake of *E. coli* into root cells. As the way we look at these interactions changes, it can be possible to get a detailed explanation of the mechanisms that assist in plant growth promotion.

5.3.1.2 Hormonal stimulation

The production of phytohormones by rhizospheric microorganisms is regarded among the most crucial mechanisms of plant growth promotion by rhizobacteria (Spaepen et al., 2007). Phytohormones (auxins, cytokinins, gibberellins, and abscisic acid) act as chemical messengers and play a vital role in regulation of growth and development in plants (Egamberdieva et al., 2017). Although in recent years many plant genes have been reported for their transcriptional regulation by indole acetic acid (IAA), however, our understanding of the microbial auxin signal transduction pathway(s) in plants is yet to evolve. Microorganisms modulate plant hormonal level and govern the plant growth leading to diverse outcomes from pathogenesis to promotion of plant growth, similar to exogenous applied phytohormones (Turant et al., 2014; Shahzad et al., 2016). The positive and negative effects of exogenous auxin on plant development is depending upon two factors, firstly the amount of IAA produced and available to the plant, and second the sensitivity of the plant tissue to variations in IAA levels. This effect of IAA is well-known in case of phytopathogenic bacteria like *Agrobacterium* spp. and *Pseudomonas savastanoi* pv. *savastanoi*, and plant growth promoting rhizobacteria (PGPR) such as *Azospirillum* spp. (Spaepen et al., 2007). The studies reported that plant become more susceptible to the bacterial pathogen and disease symptom development on exogenous application of auxin. It can be speculated that the property of bacterial pathogens to produce IAA is part of the strategy used by them to circumvent the plant defense system (Jones and Dangl, 2006; Chen et al., 2007). Khare and Arora (2010) showed the IAA produced by plant growth promoting rhizobacteria induce development of roots system, providing nutrients and support to the plants infected by *Macrophomina phaseolina*, hence suppressing the charcoal rot disease of chickpea.

Auxin produced by rhizobia is a part of many processes of root nodule development in legumes, beginning from founder cell specification (auxin transport inhibition mainly by flavonoids), nodule initiation and differentiation (due to accumulation of auxin), vascular bundle formation, and also determine the nodule numbers (long distance auxin transport). Because many rhizobacteria have the potential of producing IAA through different pathways, it is regarded that bacterially produced auxin may change the auxin balance inside the plant and influence the plant physiology. Nodules developed by *Bradyrhizobium japonicum* mutant that overproducing IAA, contain elevated levels of IAA (Hunter, 1989). The Nitrogen fixing ability in the nodules developed from IAA overproducing rhizobia was also improved (Camerini et al., 2008). *Sinorhizobium meliloti* strain over expressing IAA biosynthetic pathway exhibited raised tolerance to several abiotic stresses. Higher IAA content in nodules and roots is resulted by the inoculation of *Medicago truncatula* plants with this strain and is better resistant to salt stress (Bianco and Defez, 2009). A huge change in protein levels was observed in *M. truncatula* treated with either auxin or *S. meliloti*, most likely due to the regulation of these proteins by auxin during the initial stages of nodulation (van Noorden et al., 2007).

It is well known that cytokinins produced by PGPR also have role in their ability of plant growth enhancement (Arkhipova et al., 2005). Genetic basis and signal transduction components that mediate the beneficial effects of cytokinins on plants is still quite unexplored. López-Bucio et al. (2007) provided a crucial knowledge; how cytokinin from *Bacillus megaterium* played an important role in growth enhancement of bean (*Phaseolus vulgaris* L.) plants. Inoculation with this bacterium altered the root system architecture in *Arabidopsis thaliana* and bean plant, more precisely inhibited the primary root growth and enhanced the lateral root formation and root hair length, responsible for observed increase in biomass.

Several other studies also provided the series of evidences that phytohormones produced by microorganisms regulate the metabolism of endogenous growth regulators of plants (Hashem et al., 2016; Sorty et al., 2016) and perform a major role in transforming root morphology upon exposure to drought, salinity, extreme temperature and heavy metal toxicity (Spaepen et al., 2008; Khan et al., 2011; Tyagi et al., 2018). It is interesting to know that hormones produced by microbes can cross-talk with other phytohormones and elicits important responses (Figueiredo et al., 2008; Egamberdieva et al., 2017).

5.3.1.3 Other signals

Plants have the ability to sense microbe or pathogen-derived compounds and adjust their defense or growth responses according to the type of microorganism encountered (Khare et al., 2018). Elicitors are molecules derived from beneficial or pathogenic microorganisms play foremost role in plant defense responses (Hammond-Kosack and Jones, 1996; Mackey and McFall, 2006). The plant innate immune systems pattern recognition receptors (PRRs) perceive elicitors molecules common to different categories of microbes known as microbe-associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs) (Mackey and McFall, 2006). MAMPs act as general elicitors in plants and play a part in non-specific immunity effective against a broad range of pathogens (Jones and Dangl, 2006). Biochemically MAMPs belong to either proteins, glycans or lipids. Certain beneficial bacteria produce rhamnolipids a type of biosurfactant, which are unique MAMPs conferring resistance to pathogenic fungi in grapevine (Varnier et al., 2009).

Acylated homoserine lactones (AHLs) and volatile organic compounds (VOCs) are other signal molecules from microbes that too play an important role in plant morphogenetic processes. These are common bacterial QS compounds, which regulate gene expression in population density dependent manner. Now it is well known fact that microbial AHLs can be recognized by plants and alters their gene expression, hence modulate defense and cell growth responses of plant (Ortíz-Castro et al., 2009).

Volatiles produced by certain PGPR such as acetoin, 2,3-butanediol and dimethyl disulfide might be employed in plant-bacteria communication, and act as trigger for plant growth promotion (Ortíz-Castro et al., 2009; Meldau et al., 2013). VOCs from *Bacillus amyloliquefaciens* IN937a triggered induced systemic resistance (ISR) via an ethylene-independent signaling direction, while VOCs from another strain GB03 appear to work through an ethylene-dependent pathway, independently from salicylic acid and jasmonic acid signaling pathways. This finding provided new insight into the role of depth view of VOCs as stimulants of defense responses in plants (Ryu et al., 2004). Root exudate from elicited plants contains an array of compounds that not detected in the banquet of non-elicited plants. Exogenous treatment of plants with elicitors or other molecules from defense signaling pathways, such as salicylic acid, methyl jasmonate and nitric oxide also stimulates the buildup of an extensive array of secondary metabolites including indole glucosinolates, phytoalexins and alkamides (Noritake et al., 1996; Brader et al., 2001). These molecular interactions decide the ultimate result of the relationship, ranging from pathogenesis to symbiosis, generally via extremely coordinated cellular processes (Bais et al., 2004).

The term “induced-systemic tolerance” (IST) was coined for PGPR-induced physical and chemical changes in plants responsible for enhancement in abiotic stress tolerance (Yang et al., 2009). Zhang et al. (2008) during their study on salt stress evaluated the IST effect of VOCs produced by *Bacillus subtilis* GB03 in *Arabidopsis*. Banchio et al. (2009) reported remarkable effect of VOCs on secondary metabolite accumulation in plants responsible for aroma and flavor in agronomic crops. Early studies on IST to drought reported that inoculation of pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) plants with 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing *Achromobacter piechaudii*, conferred IST to drought stress (Mayak et al., 2004). Figueiredo et al. (2008) exhibited the consequence of drought stress on bean (*Phaseolus vulgaris* L.) was reduced when coinoculated with *Rhizobium tropici* and two strains of *Paenibacillus polymyxa*. It would be interesting to know how abiotic stress (drought/ temperature/ moisture etc.) affects the abundance or performance of rhizospheric bacteria; and play role in plant interactions. Regardless of these reports which were published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess. More recently, Tyagi et al., (2019) have reported that exogenous volatile dimethyl disulfide can alter the root system architecture in arabidopsis plants by modulating canonical auxin signaling pathway. Further researches are required to elucidate the signal transduction pathways that result from treatment of plants with PGPR under stress conditions.

5.3.2 Pathogenic interactions

In nature, a co-evolutionary relationship exists between plants and their pathogens. The host and their pathogen species contribute reciprocally to the forces of natural selection they exert on each other (Begon et al., 1996). Bacteria, fungi, oomycetes, and nematodes are common plant pathogens ensures their parasitic colonization through secretion of proteins and other molecules collectively known as effectors to different cellular compartments of their hosts, and grudgingly also modulate plant defense system (Abramovitch et al., 2006; van der Hoorn and Kamoun, 2008). Understanding the molecular function of these effectors is commonly regarded as critical for a mechanistic understanding of the processes underlying host colonization and pathogenicity. The term “effector” became popular in the field of plant-microbe interactions includes many molecules, such as MAMPs, PAMPs, toxins, and degradative enzymes. Until the precise role of pathogen molecules are revealed, it would be appropriate to call them effectors. Thereafter they can be renamed accordingly to their activities.

Gram-negative bacteria use specific secretory systems, such as type III secretion system (T3SS), to deliver effector proteins inside host cells (Abramovitch et al., 2006; Zhou and Chai, 2008). Biotrophic fungi and oomycetes have evolved haustoria to transfer pathogenicity factors into the host cell during infection process (Dodds et al., 2009). Some necrotrophic fungal proteins, notably the *Pyrenophora tritici-repentis* host-selective toxin ToxA, might not need the pathogen for translocating inside plant cells (Manning and Ciuffetti, 2005). ToxA moves inside host cells presumably by means of a plant surface receptor, target photosynthetic machinery, enhance reactive oxygen species (ROS) accumulation and kill the plant (Manning et al., 2008). Some effectors for example produced by *Cladosporium fulvum* a pathogen of tomato, act extracellularly at the plant-microbe interface and obstruct apoplastic plant defenses (Kamoun, 2006; Misas- Villamil and van der Hoorn, 2008).

Repression of plant innate immunity is the prime role of T3SS effectors of plant pathogenic bacteria (Abramovitch et al., 2006; Zhou and Chai, 2008; Pelgrom and Ackerveken, 2016). Some of the effectors found to alter host plant morphology and behavior. An example is the expression of *Xanthomonas citri pthA* in citrus cells causes development of canker, the symptom appeared as macroscopic hyperplastic lesion (Duan et al., 1999). These cankers are thought to enhance bacterial dissemination through facilitation of bacterial release from infected tissue. Many plant-associated organisms lead to development of structural malformations either in the form of protective niche or to enhance their dispersal. The most common examples include root nodules developed by rhizobia (Oldroyd and Downie, 2008), and crown galls induced by *Agrobacterium* spp. (Chalupowicz et al., 2006). Surprisingly, many effectors produce analogs and mimics of plant molecules. Many plant pathogens produce hormones viz., auxins and cytokinins as formed by plants showing phenomenon of phytohormone mimicry (Costacurta and Vanderleyden, 1995). Hogenhout and Loria (2008) reported that *fas* operons of *Rhodococcus fascians* and *Streptomyces turgidiscabies* produce cytokinins. Several fungi are known to produce gibberellins such as *Gibberella fujikuroi* (Kawaide, 2006), a reputed cause of foolish seedling disease of rice (Tudzynski, 1999). In spite of attention from at least three quarters of a decade on the importance of pathogenic interactions, a common basis with respect to knowledge and analytical guidelines is still absent. The nature does not work with pure cultures alone but most frequently with associations. The studies of multiple diseases from various plant-pathogen systems, which showed that interaction was evident among pathogens (Xu et al., 2005), encourages the continued research on this subject.

5.4 Microbe-microbe interaction

5.4.1 Intraspecies signaling

Bacteria interact via secretion of small diffusible signaling compounds, autoinducers during growth. These autoinducers gather in the area surrounding the bacteria and after reaching optimum concentration, activate the expression of genes important for bioluminescence, virulence, antibiotic production, competence, and sporulation (Kaiser and Losick, 1993; Salmond et al., 1995). Such regulation is known as “quorum sensing” (QS) because alterations in behavior need a high localized concentration of signal-producing bacteria, i.e., a quorum, to buildup strong signal to activate specific receptors in the bacteria. This starts cascade of proceedings leading to regulation of the transcription of a number of genes in bacteria. Lately, QS was regarded as a special quality shown by bacteria but studies conducted recently showed that pathogenic polymorphic fungus *Candida albicans* also showed QS phenomena (Padder et al., 2018). Till today, many categories of extracellular signal molecule have been reported in bacteria (Table 5.1). Most QS signals are either small organic molecules of ~ 1000 Da or peptides of 5–20 amino acids (Chhabra et al., 2005; Williams et al., 2007). Some common signal molecules utilized by Gram negative bacteria are N-acylhomoserine lactones (AHLs), 2-alkyl-4(1H)-quinolones (AQs), long-chain fatty acids and fatty acid methyl esters as well as autoinducer-2 (AI-2), a collective term for a group of inter-convertible furanones derived from dihydroxypentanedione (DPD). AI-2 is also produced by some

TABLE 5.1 Signaling molecules of the QS system in plant associated microbes.

S. No.	Signaling molecule	Producing species	References
1	Acylated homoserine lactones (AHLs)	Many Gram-negative bacteria	Patel et al., 2016; Yang et al., 2018
2	2-alkyl-4(1H)-quinolones	<i>Pseudomonas aeruginosa</i>	Niewerth et al., 2011; Rampioni et al., 2016
3	Autoinducer-2	Many Gram negative, Gram positive bacteria and Oomycetes	Vendeville et al., 2005; Kong et al., 2010; Lu et al., 2017
4	Bradyoxetin	<i>Bradyrhizobium japonicum</i>	Loh et al., 2002
5	Butanolides	<i>Streptomyces</i>	Yamada et al., 1987
6	Cyclic dipeptides (CDPs)	<i>P. fluorescens</i> , <i>P. alcaligenes</i>	Degrassi et al., 2002; Bellezza et al., 2014
7	Fatty acid derivatives	<i>Ralstonia solanaceum</i>	Flavier et al., 1997
8	Diffusible signal factor	<i>Xanthomonas campestris</i>	Wang et al., 2004; Tewari and Arora, 2013
9	Indole and derivatives	Many Gram-negative and Gram-positive bacteria	Kim et al., 2011; Lee et al., 2015
10	Short peptides	Gram-positive bacteria	Clewell and Weaver, 1993; Asad and Opal, 2008

Gram-positive bacteria; however, usually these microorganisms prefer linear, modified or cyclic peptides such as the autoinducing peptides (AIPs) (Xavier and Bassler, 2003). Kong et al. (2010) reported production of AI-2 by fungi as well. Additional structural classes of signal molecules such as indole and its derivatives have also been described to produced and utilized by microorganisms (Kim et al., 2011).

5.4.1.1 *N*-Acyl-*L*-homoserine lactones

Among bacteria *N*-acyl-homoserine lactones (AHLs) are a vital class of signaling molecules involved in communication between cells (Ortíz-Castro et al., 2009). AHLs is made of a conserved homoserine lactone (HSL) ring with an amide (N)-linked acyl side chain range from C4 - C18 in length. The variation in structure of different AHLs can be seen in acyl side chain in form of 3-hydroxy or a 3-oxo group. The type of AHL produced by a particular species is often strain-dependent (Ortíz-Castro et al., 2009). LuxI-type AHL synthases with LuxR-type transcriptional regulator proteins involved in synthesis of AHL molecules. The expression of these specific target genes is activated by LuxR-AHL complex (Lazdunski et al., 2004). A good number of species of bacteria respond only to their cognate molecule(s), contributing a certain degree of specificity, and hence AHL signaling is usually of an intraspecies nature (Diggle et al., 2007). However, some bacteria are competent to utilize AHLs synthesized by other species. Several excellent reviews on AHL-mediated gene regulation is published based on AHL mediated gene regulation (Waters and Bassler, 2005; Jayaraman and Wood, 2008; Ng and Bassler, 2009; Papenfort and Bassler, 2016).

5.4.1.2 *Quinolone signal*

2-Heptyl-4(1H)-quinolone and its 3-hydroxy derivative are produced by bacteria as secondary metabolite, well-known to show many biological properties. Quinolones act as signaling molecule and play important part in the QS system of *Pseudomonas aeruginosa*, termed the “*Pseudomonas* quinolone signal” (PQS). During last decades PQS have received extensive consideration (Niewerth et al., 2011; Rampioni et al., 2016). PQS is synthesized from anthranilate (Calfee et al., 2001) by the products of the *pqsABCDEH* operon. The expression of operon *pqsABCDE* is under regulation of a LysR-like transcriptional regulator PqsR (Calfee et al., 2001; Dandela et al., 2018; Lin et al., 2018). PQS is mostly synthesized during the late stationary phase of growth (McNight et al., 2000). However, during particular conditions, the production of PQS starts at the onset of stationary phase (McGrath et al., 2004). The cascade of molecular events that initiated by PQS to control gene expression is still unidentified.

5.4.1.3 *Autoinducer-2*

Another important class of signaling molecule is autoinducer-2 (AI-2) which is reported to be produced by more than 30 bacterial species. AI-2 is shown to alter the inter- and intra-specific behavior, however exact role of AI-2 in bacterial signaling currently is not much clear (Xavier and Bassler, 2003; Lu et al., 2017). Synthesis of AI-2 involves the products of the *pfs* and *luxS* genes. AI-2 are known for the involvement in the activated methyl cycle, and bacteria can also use it as a nutrient source (Federle, 2009). AI-2 influenced the expression of several genes involved in virulence, motility, biofilm formation and biosurfactant production by *P. aeruginosa* (Pierson and Pierson, 2007; Federle, 2009). The production of AI-2 by members of Pythiaceae in the eukaryotic Stramenopila kingdom, such as *Phytophthora nicotianae*, *P. sojae* and *Pythium aphanidermatum* was first reported by Kong et al. (2010). They suggested that zoosporic Oomycetes may form 4,5-dihydroxy-2,3-pentanedione using central intermediate ribose-5-phosphate. Although, it is not clear whether, Oomycetes use AI-2 as a mode of communication within the population and coordinate virulent behavior such as aggregation and plant infection.

5.4.1.4 *Auto-inducing peptides*

Autoinducing peptides (AIP), are cyclic peptides produced by Gram positive bacteria for intercellular communication, contains a high-energy thiolactone linkage (Wang et al., 2015). Most AIP signal molecules are produced from larger precursor peptides through cleavage and subsequent modifications that include substitution with isoprenyl groups, formation of lactone and thiolactone rings and lanthionines (Mayville et al., 1999; Nakayama et al., 2001; Ansaldi et al., 2002). Release of AIP from the cell takes place through oligopeptide exporters. At threshold concentration of AIP molecules, signal perception is mediated by sensor histidine kinases located in the cytoplasmic membrane. AIPs are also sought to act as an antimicrobial peptides (Verbeke et al., 2017). Sturme et al. (2002) reported that AIP from *Lactococcus lactis*, have antimicrobial properties while De Spiegeleer et al. (2015) and Wynendaele et al. (2015)

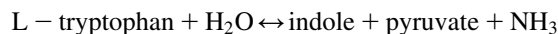
investigated the role of bacterial AIPS on human tumor progression. Other biological actions of AIPS are being explored as well.

5.4.1.5 Cyclic dipeptides

Cyclic dipeptides also known as diketopiperazines (DKPs), are a collection of signaling molecules that are used by bacteria to communicate information about population size and to regulate a behavioral switch from symbiosis with their host to virulence (Bellezza et al., 2014). Holden et al. (2000) originally detected cyclic dipeptides culture supernatants of *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* and reported its influence on N-AHL-dependent quorum sensing system (Holden et al., 2000). These peptides can mimic the function of AHL and interact with LuxR proteins at, or near, the N-AHL binding site (Degrassi et al., 2002). However, the concentrations of DKPs required to activate AHL biosensors is about 0.3 mM, much higher than the levels of N-AHL (1 nM) required to activate LuxR protein (Lazazzera and Grossman, 1998). DKPs produced from *Streptomyces* sp. and *Bacillus* spp. also have been reported to participate in communication with plants and having antibacterial and antifungal activities (Kumar et al., 2014; Wattana-Amorn et al., 2016).

5.4.1.6 Indole

Indole is well-known microbial compound produced from both Gram-positive and Gram-negative bacteria (Lee et al., 2015). Indole and its derivatives are prevalent among plant and soil-associated bacteria (Patten and Glick, 2002; Theunis et al., 2004), and also produced by some human and plant pathogens (Domergue et al., 2005; Lee et al., 2007a). Tryptophanase enzyme (TnaA; EC 4.1.99.1) of these microorganisms reversibly convert tryptophan into indole, pyruvate, and ammonia according to reaction below (Newton and Snell, 1965).



In Gram negative bacteria indole controls the virulence, drug and acid resistance, biofilm formation and several other biological functions in microbial community (Lee et al., 2007b; Nikaido et al., 2008; Hirakawa et al., 2009; Hirakawa et al., 2009). Indole also increases sporulation through indole binding pyruvate kinase as reported in case of Gram-positive bacteria *Stigmatella aurantiaca* (Stamm et al., 2005).

5.4.1.7 Short peptides

Streptococcus and *Bacillus* are the common Gram positive bacteria that especially communicate through short peptides. These short peptides frequently contain chemical modifications. For example, the signaling molecule ComX produced by *B. subtilis* for genetic competence is a 6 amino acid peptide in which tryptophan residue has been modified by the a geranyl group (Okada et al., 2005). Signaling peptides such as ComX, when reach a certain threshold concentration in environment detected by membrane bound two-component sensor histidine kinases. This recognition step is followed by signal transduction through phosphorylation cascades (Kleerebezem and Quadri, 2001). This ultimately impinges on DNA binding transcription factors responsible for differential expression of target genes.

5.4.1.8 Diffusible signal factors

Diffusible signal factors (DSF) are small molecules or signals used by bacteria to coordinate within the population, regulate phenotypic expression, and help to compete in natural environment (Tewari and Arora, 2013). In *Xanthomonas campestris*, cell to cell communication intervene through DSF that controls virulence factor synthesis and virulence to plants (Barber et al., 1997). DSF is a structural analog of *cis*-11-methyl-2-dodecenoic acid (Wang et al., 2004), synthesized and perceived by the products of the *rpf* gene cluster. DSF synthesis requires two proteins, RpfF with some amino acid sequence homologous to enoyl coenzyme A hydratases and RpfB, a long-chain fatty acyl coenzyme A ligase (Barber et al., 1997). Recognition of DSF involves a two-component regulatory system, comprising of sensor RpfC and response regulator RpfG (Slater et al., 2000). Homologues of Rpf proteins are extensively present in xanthomonads, such as *Xylella fastidiosa* and other plant pathogenic *Xanthomonas* spp. Various functions in these bacteria are under the control of *rpf*/DSF system, for example virulence, virulence factor synthesis, aggregative behavior and biofilm formation (Fouhy et al., 2007; Chatterjee et al., 2008).

5.4.1.9 Other signal molecules

Apart from AHL, other quorum sensing signaling molecule like butyrolactone have been studied extensively in *Streptomyces* spp. These signals control morphological differentiation and secondary metabolite production in bacteria (Horinouchi, 1999; Chater and Horinouchi, 2003; Kato et al., 2007). Until now almost 15 bacterial butyrolactones have been identified from different *Streptomyces* spp. (Biarnes-Carrera et al., 2015). Legume root-nodulating bacteria (rhizobia) generally utilize quorum sensing signal AHLs to regulate the induction of gene expression. However, in case of *Bradyrhizobium japonicum*, bradyoxetin controls the nodulation genes expression in quorum sensing manner. The production of bradyoxetin increases in iron depletion condition (Loh et al., 2002; Loh and Stacey, 2003). At high cell densities bradyoxetin accumulates in high concentration in immediate environment leading a cascade of molecular events initiated by the regulator NswB, which ultimately represses the *nod* genes expression (Jitackorn and Sadowsky, 2008). Bradyoxetin has also been found in other Alphaproteobacteria, but its function in these species is still unknown (Loh et al., 2002; Loh and Stacey, 2003). Another signal molecule is the long chain fatty acid derivative, 3-hydroxypalmitic acid methyl ester produced by *Ralstonia solanacearum* (Flavier et al., 1997; Lee et al., 2018). Moreover, long chain fatty acids is another class of signals that also serve in prokaryotic differentiation systems, indicating that this category of molecules could be wide spread in bacteria.

Winzer et al. (2002) anticipated four criteria to describe signaling molecules involved in intraspecies communication. They explained specific issues of the production, perception (the signal molecules accumulates extracellularly and is recognized by a specific receptor), cellular sensitivity (a concerted response generates once signal molecule accumulates to the critical threshold concentration) and cellular response to the signal extends beyond physiological changes needed to be metabolized or detoxified. Following these criteria, the above described signal molecules can be considered as intraspecies in at least one organism.

5.4.2 Interspecies and interkingdom signaling

In complex ecosystems, microbes must simultaneously exchange signals with members of their own species as well as of other genus, and even with members of other kingdom. Most of the available information regarding production, perception and function of QS signal molecules concerns the interactions within population of single species; however, very few attentions was received by microbial consortia. A signal, in this context, is a molecule generated by one organism that is sensed by another, resulting in modulation of cellular response by change in gene expression (Dunn and Handelsman, 2002). Among QS molecules AHL of same type was known to produce by different genera capable of cross-talk (Arora et al., 2010). For example, N-butanoyl homoserine lactone (C4-HSL) was produced by *Pseudomonas aeruginosa*, *Serratia liquefaciens* and *Aeromonas hydrophila* (Eberl et al., 1996; Swift et al., 1997).

Members of the legume nodulating bacteria (rhizobia) produce signal molecules of diverse types. Some rhizobia produce only one type of signal molecule, while others known for production of as many as nine alleged signals (Cha et al., 1998). 3-oxo-C8-HSL, a type of AHL is common among many rhizobial species such as *Rhizobium fredii*, *Rhizobium leguminosarum* bv. *viciae*, bv. *phaseoli* and bv. *Trifolii*, *Sinorhizobium meliloti*. Extremely nonpolar compounds with long acyl side-chains are also produced by several rhizobia (Sanchez-Contreras et al., 2007). Interspecies interactions of either synergistic or competitive nature play an imperative function in the dynamics of these microbial communities (Marketon and González, 2002).

Genes for AI-2 production present in both Gram-negative and Gram-positive bacteria. This notion has created a hypothesis of 'Bacterial Esperanto' that AI-2 is employed as interspecies communication signal (Winans, 2002). Zoospore pathogens belonging to Pythiaceae were reported to produce AI-2, indicates the possibility of AI-2 used as a common signal of communication with bacteria (Kong et al., 2010). The ability of these pathogens to survive in soil with plethora of microorganisms and to tolerate recurrent culture contamination by bacteria appears to be prearranged by these interkingdom interactions. It will be fascinating to authenticate if this cross-kingdom communication is bridged by AI-2.

Diffusible signal factors (DSF) are another group of signal factors found to be act as interspecies signal. *Burkholderia cenocepacia* produces a type of DSF, cis-2-dodecenoic acid (BDSF), a structural analog of cis-11-methyl-2-dodecenoic acid synthesized by *Xanthomonas campestris*. Boon et al. (2008) reported that BDSF restores biofilm production in *X. campestris* DSF-deficient mutants. BDSF was also found to inhibit the growth of *Candida albicans* (Boon et al., 2008). Shank and Kolter (2009) during their study found that cis-2-decenoic acid produced by *Pseudomonas aeruginosa* is structurally related to DSF and BDSF (Shank and Kolter, 2009). Cis-2-decenoic acid is known for the ability of biofilm dispersion from a various type of microorganisms including *Escherichia coli*, *Streptococcus pyogenes*,

B. subtilis, *C. albicans* etc. along with *P. aeruginosa*, and also prevents biofilm formation to certain level (Shank and Kolter, 2009; Maxwell et al., 2016). These findings reported the possibility of crosstalk between related signaling systems. However, there is still much to explore how related signals and receptors are involved in interspecies communication.

Antibiotics are low molecular weight (<3000 Da) organic molecules produced by microorganisms that have been purified for their ability to inhibit or kill bacteria. However, another possible role as signaling compound is quite ignored. As the bacteria causing infectious diseases in soil is low, nowadays an emerging concept is that antibiotics are not solely bacterial weapons but at sub-inhibitory concentrations can reduce the bacterial fitness and virulence by acting as interspecies signaling molecules. At below threshold levels they may modulate the expression of 5–10% genes of bacteria (Goh et al., 2002; Linares et al., 2006). *Pseudomonas aeruginosa* and *E. coli* act in response to subinhibitory levels of aminoglycosides and develop antibiotic-resistant biofilms (Hoffman et al., 2005). This appears that Gram negative bacteria use this as a strategy to counteract against antibiotic producing Gram-positive soil bacteria such as the streptomycetes. Khare and Arora (2011) reported the purified pyocyanin from *Pseudomonas aeruginosa* act as signaling molecule and induce the development biofilm by rhizobia. This can be an answer to the reports on better root nodule development in legumes by coinoculants of *Pseudomonas* sp. and rhizobia over monoinoculants of rhizobia (Arora et al., 2008; Khare and Arora, 2011).

Recent reports indicate that not only eukaryotic organisms like fungi or plants but bacteria could also disrupt AHL signaling by degrading AHLs by a process called quorum quenching (Wong et al., 2013). It is interesting to know that AHLs producing bacteria bears genes for enzymes that degrade AHLs. These enzymes from *P. aeruginosa* PAO1 (*pvdQ*) and *Agrobacterium tumefaciens* (*attM* and *aiiB*) possibly play an important function in regulating quorum sensing (Carlier et al., 2003; Huang et al., 2003). On the other side, AHL-degrading enzymes were also synthesized by AHL-nonproducing bacteria probably to be used as a defense mechanism to compete with antibiotic-producing bacteria in their natural habitat or to control plant pathogens by PGPR. A *Bacillus* isolate contains *aiiA*, a gene that encodes a 250-amino acid AHL-lactonase protein that hydrolyzes the lactone bond of AHLs (Dong et al., 2000, 2001). Transgenic tobacco and potato expressing *aiiA* were more resistant to disease caused by *Erwinia carotovora*. The possible reason behind increased resistance is the disruption of quorum sensing in *E. carotovora* (Dong et al., 2000). The oomycete *Pythium ultimum* represses genes expression in *Pseudomonas fluorescens*, that consequentially leads to alteration in establishment of bacterium in ecological niche (Fredri et al., 1997). Repression of *rrn* operons by *P. ultimum* results in reduced growth rates, and this repression could be an effectual tactic for preventing the colonization of biocontrol strain in the rhizosphere (Smith et al., 1999). Tannières et al. (2017) reported that production of QS degrading enzymes by quorum sensing signal molecules producing populations can serve as a strategy to combat QS-cheating and QS-hijacking. Their experiment with *Agrobacterium tumefaciens* showed that expression of QS disrupting enzyme(s) controls the signal molecules utilization by QS signal-negative mutants that eventually stops the dissemination of QS signal negative Ti plasmids. Thus multifaceted communications that contribute to or detracts from successful disease suppression offers a model for understanding communication networks and also will contribute to our skills to stimulate and enhance the efficacy of biocontrol.

5.5 Conclusion

It is clear from the above discussion that our understanding of root-mediated processes has crossed the boundaries of the classical belief that the only functions of roots are anchorage and uptake of water and nutrients from soil. Now another exclusive role of roots is come to light, that roots are rhizosphere ambassadors, assisting cross-communication between the plant and microorganisms. The plant growth and productivity is an outcome of these dynamic interactions and communication between the root and microorganisms. Earlier studies reported that the population of AHL-producing bacteria in the rhizosphere associated with plants is higher over the bulk soil. In nature microorganisms exists as mixed populations, where biofilms development, maturation and their dispersal are potentially to be tightly regulated through cooperative intraspecies, interspecies and interkingdom signaling. Given the vast number of studies on plant microbe-microbe interactions, the implementation of this knowledge in agriculture is possibly representing only the ‘tip of the iceberg’. Advanced researches on the molecular nature of plant-microbe cross-talks are now just being implemented to improvise methods for protection of plants from infectious diseases. A burning confront for microbiologists of the 21st century is to design a virtual system of the communication networks that connection together microorganisms and plants in rhizosphere communities. The synopsis of these researches indicates that that, in the future, plant disease control basic procedures i.e., implementation of chemicals, mainly fungicides and bactericides, will be complemented or reinstated by new technologies emerging from the advanced knowledge of plant-microbe-microbe interactions.

However, comparative studies are required to fully understand pathogenic and commensal interactions in mixed populations with the same plant. Collaboration between plant biologists, ecologists, and soil scientists is the need of future world for the development of effective and ecofriendly agro-technologies.

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Chapter 6

Application and biological impact of endophytic bacteria as IAA producers

Ewa B. Moliszewska and Małgorzata Nabrdalik

Faculty of Environmental and Technical Sciences, Institute of Environmental Engineering and Biotechnology, Opole University, Opole, Poland

Chapter outline

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6.1 Introduction

Endophytes are well defined by Le Cocq and coworkers (as cited in [Apine and Jadhav, 2011](#)) as “microbes which occur within plant tissue for at least part of their life cycle without causing disease under any known circumstances”. The first definition was created by de Bary, who considered any organisms occurring inside plant tissues. Other definitions were focused only on fungal endophytes or bacterial ones, but it is clear that all of them should be considered as a habitat, not a function, and should include all organisms which colonize internal plant tissues during part or all life cycle. The result of colonization refers to both, plant host and microbial endophyte ([Apine and Jadhav, 2011](#); [Hardoim et al., 2015](#)). The crucial feature for endophytes is lack of the ability to cause any plant disease, although they have huge influence on plants’ lives. This is why they are intensively studied for searching of their beneficial role in plants. One of those most interesting features of bacterial endophytes is the possibility to produce IAA. IAA is a common abbreviated name for indole-3-acetic acid. It is an important molecule acting as phytohormone, although not only plants are able to produce it. Plants’ growth is connected in a natural way with many microorganisms, including pathogens and endophytes. Because IAA controls plant growth and development not only in a beneficial way, but also in pathogenic processes, therefore bacterial and fungal pathogens are able to synthesize the molecule. Some of endophytes play an important role in plants’ growth and protection against pathogens, that’s why they are intensively investigated and applied in agriculture. The auxin-signaling pathways in plants, which regulate organogenesis, growth, and environmental responses, are transcriptionally controlled by Aux/IAA genes. A group of 29 Aux/IAA genes was recognized in *Arabidopsis*. They exhibit unique and partially overlapping expression patterns, although the Aux/IAA genes have some specialized function. Proteins coded by them are auxin-sensitive repressors important in diverse physiological processes in plants’ development. Regulation of Aux/IAA expression processes is little known, although some factors are well described. [Shani et al. \(2017\)](#) described transcription factors from 38 families that directly promoted transcription of the IAA5 and IAA19 genes in reaction to abiotic stress. The recessive mutations that they obtained in these IAA genes resulted in decreasing tolerance to environmental stress conditions, showing a crucial role for auxin in abiotic stresses. This show why Aux/IAA genes are integrative points for genetic and environmental information influencing plant development and physiology ([Shani et al., 2017](#)).

6.2 Biosynthesis of IAA by bacteria

As the first nineteenth century botanical observations gave a potential knowledge about plant growth regulations, the next investigations showed a complicated nature of IAA biosynthesis with presence of multiple redundant and parallel pathways even in a single organism. The pathways may create a multi-compensate system what makes plants to be impossible to develop as IAA auxotrophic mutants completely devoid of the possibility to IAA producing (Lehmann et al., 2010; Duca et al., 2014). Aromatic amino acid aminotransferase (ArAT) is one of the compounds involved in IAA biosynthesis, and catalyses the reaction of reversible transamination between aromatic amino acids and 2-oxoglutarate. It is an enzyme usually occurring in bacteria in the form of several isozymes. This enzyme has different metabolic functions and participates at least in six metabolic pathways (Mavrides and Orr, 1975; Pedraza et al., 2004), but is also examined due to its involvement in the IAA biosynthetic pathway that occurs in many bacterial species (Szkop et al., 2012).

Bacteria being able to produce IAA are members of endophytic biota as well as free-living soil biota, epiphytic biota and water-living biota. Most of IAA producing and plants' associated bacteria are members of the several phyla: *Proteobacteria*, *Bacteroidetes* *Actinobacteria*, *Firmicutes* (Nabrdalik et al., 2018). In bacterial biosynthetic pathways tryptophan may be involved in IAA production or it may be produced independently of tryptophan. The main tryptophan utilizing pathways are recognized as: (I) indole-3-pyruvic acid (IPA) pathway, initiated by aminotransferase (L-Trp transaminase); (II) indole-3-acetamide (IAM) pathway catalysed by Trp-2-mono-oxygenase and indoleacetamide hydrolase, and (III) indole-3-acetaldehyde (IAAld) pathway. In the last one pathway, tryptophan is converted directly by tryptophan side chain oxidase (TSO). Alternatively tryptophan is converted via IPA into IAAld by aminotransferase, indole acetaldehyde dehydrogenase, mutase or oxidase enzymes (Oberhänsli et al., 1991; Duca et al., 2014).

The overview of synthetic pathways of IAA seems to show their dual character; while the IAM pathway is more characteristic for phytopathogens, the IPA pathway is rather associated with plant growth promoting bacteria (PGP), but also found in some phytopathogens (Glick, 2015).

Since now few genes (Table 6.1) have been well identified as involved in bacteria for IAA synthesis from tryptophan, although not each species has detailed identified enzymatic activity and involved pathway. Bacteria may possess

TABLE 6.1 Genes involved in enzyme synthesis in several pathways in bacterial biosynthesis of IAA (according to Duca et al., 2014 and Greetatorn et al., 2019).

Genes	Enzymes	Pathways	Microorganisms
<i>aldA</i>	Aldehyde dehydrogenase	IPA	<i>Azospirillum brasiliense</i> Yu62
<i>hisC1</i>	Histidinol-phosphate aminotransferase	IPA	<i>Azospirillum brasiliense</i> UAP14
<i>ipdC</i>	Indolepyruvate decarboxylase	IPA	<i>Azospirillum brasiliense</i> Sp254 <i>Enterobacter cloacae</i> FERM BP-1529
<i>iaaM</i> <i>iaaM</i> , <i>iaaH</i>	Tryptophan monooxygenase Indole-3-acetamide hydrolase	IAM	<i>Pseudomonas fluorescens</i> Psd <i>Ralstonia solanacearum</i> <i>Streptomyces scabies</i>
<i>tms-1</i> <i>tms-2</i>	Tryptophan monooxygenase Indole-3-acetamide hydrolase	IAM	<i>Agrobacterium</i> spp.
<i>nit</i>	Indoleacetone nitrilase	IAN	<i>Pseudomonas</i> sp. UW4 <i>Bradyrhizobium</i> sp. SUTN9-2
<i>yhcX</i>	Indoleacetone nitrilase	IAN	<i>Bacillus amyloliquefaciens</i> FZB42
<i>oxd</i>	Phenylacetaldoxime dehydratase and phenylacetyl nitrilase	IAOX/IAN	<i>Bacillus</i> sp. OxB-1 <i>Rhodococcus</i> sp. NCIMB 11216 <i>Corynebacterium</i> sp. C5
<i>oxdRC</i> <i>nha1</i>	Phenylacetaldoxime dehydratase and nitrile hydratase	IAOX/IAN	<i>Rhodococcus globerulus</i> A-4
<i>oxd</i> <i>nha1</i>	Phenylacetaldoxime dehydratase and nitrile hydratase	IAOX/IAN	<i>Rhodococcus</i> sp. NCIMB 11215
<i>oxd</i> <i>nha1</i>	Phenylacetaldoxime dehydratase and nitrile hydratase	IAOX/IAN/IAM	<i>Rhodococcus erythropolis</i> JCM 3201 <i>Rhodococcus erythropolis</i> BG 133201 <i>Rhodococcus erythropolis</i> BG 16 <i>Brevibacterium butanicum</i> ATCC 21196

several different isoform enzymes involved in IAA synthesis with all aromatic amino acids. One of the most well recognized gen is *aldA* gen known in *Azospirillum brasiliense* Yu62, *Bradyrhizobium japonicum* USDA 110 and *Mesorhizobium loti* MAFF303099, coding aldehyde dehydrogenases. Disrupting this gen resulted in mutant producing less IAA (Xie et al., 2005). The isoenzymes group was recognized also in other bacteria with IPA biosynthesis pathway of IAA. Some of those isoenzymes are highly specific for substrates, while others show broad substrate specificity, but all of them require NAD or NADP as cofactors (Duca et al., 2014). Tryptophan is deaminated to 3-indole pyruvic acid by aminotransferase, and then subsequently it is converted to indole-3-acetaldehyde (IAAld) by decarboxylase, and oxidized to IAA by aldehyde dehydrogenase, mutase, oxidase enzymes (Glick, 2015). IAAld is possible to be synthesized in two alternative pathways. One is TSO pathway with an enzyme tryptophan side-chain oxidase, and the second pathway leads through IPA pathway. TSO pathway has been recognized in *Pseudomonas fluorescens* strains (Oberhänsli et al., 1991; Duca et al., 2014). IPA pathway is common in non-pathogenic plant-associated bacteria such as e.g. *Azospirillum brasiliense* SM, but it may also be found in such phytopathogens as *Agrobacterium tumefaciens*, *P. syringae* ssp. *savastanoi* and *Erwinia herbicola* ssp. *gypsophila*. The *ipdC* gene codes indole pyruvate decarboxylase enzyme. The role of this gene was confirmed by its disruption in several bacterial strains. The enzyme belongs to the group of an α -keto acid decarboxylase with a broad substrate specificity. While the indole pyruvate decarboxylases of some bacteria show broad substrate activity, their main role is not a production of IAA, although they can play an incidental role in IAA production pathway (Duca et al., 2014; Glick, 2015). Indole-3-pyruvic decarboxylase encoded by *ipdC* gen was described for *Azospirillum*, *Enterobacter* and *Erwinia* strains. Mutants with disrupted *ipdC* gene showed lower IAA production, and presence of low IAA levels could be explained by alternative co-existing pathways (Glick, 2015).

The IAM pathway occurs most frequently in phytopathogenic bacteria with the *iaaM/tms-1* gene coding tryptophan 2-monooxygenase, and *iaaH* gene coding indoleacetamide hydrolases and amidases. Bacterial *iaaH/tms-2* gene produces hydrolases what distinguishes them from other enzymes which are able to convert more substrates than IAM pathway (Duca et al., 2014). Gene *iaaM* is also found in some isoforms, which fall into two groups; one grouping plant pathogens (*Pseudomonas savastanoi*, *P. syringae*), and the second one with organisms more like *Burkholderia* spp. – the group is not well characterized and it is not known if tryptophan is the substrate in it (Glick, 2015; Cerboneschi et al., 2016). The experiments of Manulis et al. (1998) showed that IAM pathway is more associated with pathogenic properties of bacteria (inactivation of the pathway reduced galls formation), whereas IPA pathway does not influence on gall formation by pathogenic *E. herbicola* pv. *gypsophila* if the enzymes of the pathway were disrupted.

Nitrile hydratase enzymes catalyse the hydration of indole-3-acetonitrile (IAN) into indole-3-acetamide (IAM), and then it is converted to IAA by indoleacetamide hydrolase or indoleacetamide hydratase. Thus, nitrile hydratase enzymes link the IAN pathway to IAM pathway, although they role is not exclusive for IAA production, but also they are important in biotransformation of different substrates to organic acids. They play role not only in synthesis of specific bacterial or plant substances but also serve as a way for detoxification of nitriles. In bacteria, IAN follows conversion of indole-3-acetaldoxime (IAOx) by acetaldoxime dehydratase. IAOx is converted directly from tryptophan by oxidoreductase (Prasad and Bhalla, 2010; Vejvoda et al., 2011; Duca et al., 2014).

Depending on the microbial character, there are different ways of regulation of IAA operation. Pathogenic regulation of IAA operation requires genes playing role in decreasing IAA activity. Thus, bacteria possess also genes for IAA-conjugating enzymes. Typically pathogenic enzyme has been found in *P. syringae*. It had not any essential homology with any plant IAA conjugating enzyme. Bacterial *iaaL* gene was found to encode the enzyme that conjugates IAA to lysine to create IAAeLys, but any plant hydrolases have not been discovered in case of the catabolism of IAAeLys, although several plant-associated bacteria have been shown to be able to hydrolyze plant auxin conjugates such as IAAeAsp (Cerboneschi et al., 2016).

6.3 Bacteria producing IAA

Bacteria able to produce IAA belong to numerous genera: *Aeromonas*, *Azospirillum*, *Enterobacter*, *Erwinia*, *Pantoea* and *Pseudomonas*, *Flavobacterium*, *Curtobacterium*, *Microbacterium*, *Bacillus*, *Bradyrhizobium*, *Streptomyces*, *Azotobacter*, *Klebsiella*, *Alcaligenes*, *Brevibacterium*, *Rhodococcus*, *Ralstonia*, *Corynebacterium*, *Methylobacterium*, *Dickeya dadantii* (Choudhary et al., 2007; Khan and Doty, 2009; Apine and Jadhav, 2011; Mishra et al., 2011; Brader et al., 2014; Duca et al., 2014; Pocięjowska et al., 2014; Cerboneschi et al., 2016; Santoyo et al., 2016; Nabrdalik et al., 2018; Verma and White, 2018). They live as free-soil organisms or as endophytic ones. Also pathogenic strains are involved in IAA production, although such pathogenic bacteria as *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*) and other species within the genus do not produce the compound by themselves. The production is regulated by *tms-1/tms-2* genes, that are not functional in bacteria, but switches the plant metabolism. Other plant pathogenic bacteria

like *P. savastanoi* contain *iaaM/iaaH* genes which are active in bacterium and are not transferred into plant cells, thus their pathogenic activity is a result of production of IAA, which is transferred into plant cells (Duca et al., 2014). Manulis et al. (1998) demonstrated the IPA pathway is more associated with bacterial fitness and plant colonization while the IAM pathway is rather contribute to pathogenicity.

6.4 Why the bacteria need IAA

The most important role of IAA is its' acting as a plant-microbe interaction molecule, especially that, as Kim et al. (2011a,2011b) suggested, most of endophytic bacteria are able to produce IAA. The role of IAA in improving bacterial surviving was also proved (Bianco et al., 2006). Experiments of Bianco et al. (2006) and Donati et al. (2013) showed that IAA is helpful for bacteria in persevering stressful conditions like UV, salinity, acidity, heat shock and cold shock. Bianco et al. (2009) observed that in *Sinorhizobium meliloti* IAA stimulated intracellular production of the trehalose, what is a carbon source and osmotic compound for these bacteria. Osmotic activity of trehalose endows better protection against freezing and desiccation. IAA can serve for bacteria also as a chemotaxis molecule what was observed by Scott et al. (2013), though it was finally degraded. It should be pointed that usually typical IAA degrading bacteria lack chemotaxis activity towards IAA (Duca et al., 2014).

Low IAA concentrations may regulate forming semi-mycelial structures and antibiotic activity in some *Streptomyces* strains, thus IAA secretion in rhizosphere by bacteria and plants is a signal for these *Streptomyces* spp. to enhance antibiotic production and in this way protection the niche for their life against other microbes (Matsukawa et al., 2007; Duca et al., 2014).

6.5 Methods in IAA research (isolation, detection)

First step in screening bacteria for plant growth promoting (PGP) properties is selecting them for achieving the most promising isolates with the best plant growth promoting properties as well as suitable colonization ability. Usually, typical researches are conducted in this way that bacteria colonies are taken randomly from Petri dishes. However, selection carried out randomly, may exclude some interesting bacteria in terms of their beneficial abilities for plants. Thus, it is essential to study all the isolated bacteria and select the best ones including PGP features and high colonization rate. Screening procedure should be realized in an economic and simple way (Etesami et al., 2015). The useful technique for searching rice PGP bacteria was prepared by Etesami et al. (2015), although the procedure seems to be accurate also for other plant species. The crucial in the procedure is checking the potential antagonistic activity in tested strains; this point allows avoiding future disturbances between all bacteria inoculated to plant. The procedure involves also a step as in Koch's rules, which leads to confirmation of the PGP activity. After the above mentioned step, all re-isolated bacteria should be independently tested for different beneficial activities as e.g. IAA production, ACC deaminase production, siderophore production or solubilize phosphate ability, especially that there are many evidences that IAA production may be the first signal of PGP traits. Finally the best PGP isolates have to be identified in *in vitro* tests.

As Duca et al. (2014) showed the majority of researches on biosynthetic pathways of IAA are made on bacteria. Those results were easier to obtain than researches in plants for searching the possibility of controlling genes and proteins involved in IAA synthesis (Duca et al., 2014). One of the most popular methods for determining IAA production is a colorimetric method given by Gordon and Weber (1951) modified by several researchers (Khan and Doty, 2009; Szkop et al., 2012). The method is based on the color product of the reaction of Salkowski's reagent, which originally consists of 0.5 M FeCl₃ dissolved in 35% HClO₄. At present, it is used almost exclusively for recognition and quantitative determination of IAA concentrations in bacterial culture supernatants, and also is one of the most useful methods for determination of ArAT activity (Szkop et al., 2012). It is based on the reaction of the Salkowski's reagent with indole-3-pyruvate (IPyA), which is the oxoproduct of L-tryptophan transamination. The reaction is based on modified Salkowski's reagent: 10 mmol/L FeCl₃ was dissolved in a concentrated oxidizing acid, namely in 35% v/v H₂SO₄; as Szkop et al. (2012) proved, the concentration of the reagent was the most sensitive to IPyA. The reaction mixture remains optically clear, with no pellets, after the addition of the Salkowski's reagent to the solution. The reaction of IPyA with Salkowski's reagent gives a pink colored complex and shows maximum absorbance at $\lambda = 530$ nm. The method needs a calibration curve for IPyA and Salkowski's reagent, namely 1 mL of the Salkowski's reagent has to be added to 0.5 mL of eight different concentrations of each standard solution of IPyA ranging in concentration from 3.8 to 92 μ mol/L, and prepared in 0.1 mol/L phosphate buffer containing 10 mmol/L L-tryptophan. In next step, solutions have to be mixed and incubated for 10 min in the dark at room temperature. The colored complex is stable for at least 30 min. The spectrophotometer has to be calibrated against a blank solution (without IPyA) mixed with the Salkowski's

reagent (Szkop et al., 2012). The method allows conducting quantitative examination of crude bacterial culture supernatants. For this purpose the bacteria are produced for two to seven days in the dark and sampled at intervals (hourly/daily). The possible medium is e.g. King B liquid medium or glucose peptone broth (GPB) amended with L-tryptophan (Shi et al., 2009; Szkop et al., 2012). The method allows for determining ArAT activity after disruption of bacterial cells or also it is useful for determination of IAA released by bacteria to the medium. Nabrdalik et al. (2018) for the determination of the ability to produce IAA by isolates of *P. agglomerans* have grown them, at 30 °C for 7 days, in LB medium (Laurin Broth) supplemented with L-tryptophan at concentrations of 500, 1000, 2000 µg/mL. After a proper time of incubation, samples should be purified of bacterial cells by centrifugation (e.g. 13,000 rpm for 10 min or for 20 minutes at 10,000 rpm at 10 °C.), and supernatants are measured for the presence of IAA by adding two parts of Salkowski's reagent to one part of supernatant, and followed with measuring the optical density at 530 nm after 30 min of incubation. The results have to be read from the standard curve prepared according the rules described above for IPyA, but using pure IAA dilutions. In the control trials the bacterial cultures should be grown in medium without L-tryptophan. Usually the production of IAA is measured daily, every 24 hours (Khan and Doty, 2009; Szkop et al., 2012; Nabrdalik et al., 2018).

Khan and Doty (2009) proposed another simple and effective method for assessing bacterial ability to produce IAA. They analyzed for IAA production bacterial endophytic strains using poplar (*Populus tremula* × *P. alba*) cuttings difficult to root. For this purpose, about eight cm long cuttings of poplar, taken from a glass house, were surface sterilized, then the ends of each cutting were removed and they were transferred to tubes containing ½ × Hoaglands medium and were grown for two weeks. After that time a culture (OD 600 = 0.5) of endophytic bacteria grown overnight in MG/L broth was added to tubes containing MS medium and one poplar cutting. Tubes were incubated for the next three weeks at 25 °C for 16 h/8 h of day/night photoperiod. Induction of rooting, higher growth of sprouts and greater number of new developed leaves were observed as results of IAA production during the test as compared to untreated controls.

Bric et al. (1991) described simple and rapid method, convenient to screen large numbers of bacteria if taken into consideration their ability to produce IAA. They suggest growing bacteria on medium supplemented with tryptophan (5 mM) and overlaid with a nitrocellulose membrane. Incubation should be conducted until bacterial colonies diameter reach 1–2 mm. Then membrane has to be removed and placed on a filter paper saturated with Salkowski's reagent, and incubated until (usually not longer than 1 hour) red haloes are formed around the colonies. The colorimetric reaction to IAA is sensitive to at least 50 pmol of IAA detected in 2 mm² bacterial spots (Bric et al., 1991).

TLC method for IAA recognition was described by Mohite (2013). Briefly, he suggested growing bacteria in YMD medium, mixing supernatant with ethyl acetate (1:2) and after shaking and leaving it for 10 minutes the mixture should be spotted on silica gel. As a solvent mixture for chromatogram propanol: water (8:2) is proposed. Chromatogram is developed by Salkowski's reagent. Szkop et al. (2012) described also a method for determination of ArAT activity on gels with complexing enzyme products with Salkowski's reagent, and RP-HPLC method for determination indole compounds related to bacterial IAA production (Szkop and Bielawski, 2013). The RP-HPLC method is based on fluorescent properties of indoles and provides more adequate than other methods sensitivity (0.015 µg/mL) as well as adequate sample purity. It provides also selectivity, because allows to detect seven indole compounds: indole-3-acetic acid, indole-3-acetamide, indole-3-acetonitrile, indole-3-ethanol, indole-3-lactic acid, tryptamine and tryptophan in bacterial culture supernatants. The method needs a simple sample (supernatant) filtration and is performed using a gradient elution on a symmetry C8 column followed by fluorimetric detection (Szkop and Bielawski, 2013).

6.6 Impact of presence in plants of bacteria producing IAA

Mode of action of IAA in plant organisms is due to the presence of free indole-3-acetic molecules, but lots of them are conjugated to amino acids, sugars or proteins, and thus they are not active, and plants maintain a homeostasis. As plants are a typical source of IAA, bacteria can use it as a signal molecule that is attracting them to colonize plants, and some of them may protect plants against other microbial organisms being stimulated by IAA to antibiotic production (Matsukawa et al., 2007; Duca et al., 2014; Cerboneschi et al., 2016).

The IAA production by endophytic bacteria effect depends on the level of the sum of the plants' endogenously produced IAA and IAA secreted by the endophytic bacteria, thus the summary effect can be either positive or negative (Duca et al., 2014). The effect is depended also on the character of bacterium – pathogenic one or beneficial one.

The first and main effect of IAA producing bacteria observed on plants is usually improved root development and through this better growth of the above ground parts of plants. Nabrdalik et al. (2018) observed a high IAA productivity by cultures of *Pantoea agglomerans* BC 17 and BC45 in the absence and in the presence of tryptophan, and if bacteria were used for inoculation of sugar beet seedlings, a little tendency to elongation roots of these plants was observed, but

results were not statistically significant. The positive influence of IAA produced by endophytic bacteria, including *Actinobacter johnsonii* 3-1 on sugar beet seedlings, was observed by Shi et al. (2009,2011), who obtained as a result a significant plant growth improvement and higher dry weight content. They observed very high IAA production by the strain 3-1 in third, fourth and fifth day in the medium supplemented with tryptophan, but after that time the synthesis of the auxin decreased. Shi et al. (2011) proved also that *A. johnsonii* 3-1 can significantly improve sugar beet yield by increasing sugar content, fresh mass of roots and fresh mass of leaves. Shi et al. (2010) examined some physiological parameters of sugar beet plants and discovered the possibility of promotion of the photosynthetic capacity in sugar beets infected by endophytic bacteria. These plants showed increased chlorophyll content what lead to increased carbohydrate synthesis; also parameters describing photosynthesis in these plants were significantly better than in non-inoculated ones.

Pitzschke (2016) suggested that all quinoa (*Chenopodium quinoa*) seeds are colonized by *Bacillus* spp. which promoted seeds' germination and also manipulated redox status of seedlings, increasing their vigor in stressful conditions. Shi et al. (2009) used disinfected seeds soaked in bacterial suspension for 6 h, for inoculation by endophytic bacteria, before sowing them in soil. According to Gałazka et al. (2015) seeds' inoculation of such plants as maize, wheat, barley, rye, sorghum and millet resulted in positive effects for their growth and development. Marathe et al. (2017) observed two days quickening of soya seeds germination after inoculation them by *P. aeruginosa* 6A (bc4) supplemented with tryptophan. They reported also increase in germination percent, and then increase in shoot and root length of treated by bacteria plants as compared to untreated controls. Figueira et al. (2019) proved that inoculating *Salicornia ramosissima* seeds with *Bacillus aryabhatai* SP1016-20 doubled seed germination at the high salinity, although the mechanism involved in the biocontrol test is not known.

Seedlings are the most frequently inoculated, by bio-promoting bacteria, developmental stage of plants. Many researches proved that this is a good way for the confirmation of potentially promoting bacterial activity, as well as it seems to be a good way for application purposes (Shi et al., 2009; Shi et al., 2011; Duca et al., 2014; Gałazka et al., 2015; Greetatorm et al., 2019). Soil inoculation is used less frequently as a method for inoculation and finally colonization of plants. Although Khan et al. (2014) successfully used endophytic bacteria, *Sphingomonas* sp. LK11, suspension added directly to the soil. As the result they obtained plants of tomato significantly promoted in growth (shoot length, chlorophyll contents, shoot, and root dry weights) by strain LK11, if compared to the untreated control. The strain was able to produce IAA in the amount of $11.23 \pm 0.93 \mu\text{M/mL}$, although its activity was probably due to the ability to produce gibberellins.

Verma and White (2018) observed seedlings' growth stimulation by endogenously roots' colonizing bacteria and explained the phenomena at least partly by the possibility to produce plant growth promoting hormones or/and organic or inorganic nutrient uptake from soil by developing seedlings. They discovered four types of bacteria on seeds of browntop millet (*Urochloa ramosa*): *Curtobacterium* sp., *Microbacterium* sp., *Methylobacterium* sp. and *Bacillus amyloliquefaciens*, which were able to promote seeds' and seedlings' development. They influenced on root hair formation, root and shoot length growth and photosynthetic pigments content. Two strains of bacteria, *Methylobacterium* sp. and *Bacillus amyloliquefaciens*, were definitely observed around hairy roots, within them, as well as in parenchyma and intercellular spaces of the root cortex, while two others - colonized roots' parenchyma and hairy roots. Two of them (*Microbacterium* sp. and *B. amyloloquefaciens*) were found to produce auxins. Three of them (*Curtobacterium* sp. *Microbacterium* sp. and *B. amyloloquefaciens*) showed the ability for phosphate solubilization. Marathe et al. (2017) observed higher content of N, P, K in the soil and their better assimilation by soya plants treated with *P. aeruginosa* 6A (bc4) which was supplemented with tryptophan. Those plants showed also positive effect on chlorophyll a, b content in leaves. As a conclusion they suggested that tested by them *Pseudomonas* strain would possible to be used as a bio-stimulating organism and also as a bio-fertilizer for enhancing the growth of plants.

Greetatorm et al. (2019) proved the helpful role of *Bradyrhizobium* sp. SUTN9-2 on increasing plant dry weight, chlorophyll content and vegetative growth on early seedlings' stage of rice (7 days after inoculation of one day germinated seeds). Plants showed also a relevantly higher IAA content with *Bradyrhizobium* sp. SUTN9-2 and simultaneous NH_4NO_3 treatment, when compared to untreat by endophytic bacteria plants. The phenomenon was active only in 7-th day after inoculation. Shi et al. (2011) gave evidence that endophytes may influence the plants' development and welfare for the whole life cycle; they showed significant influence of *A. johnsonii* 3-1 on the yield mass and quality of sugar beets.

Endophytes, including bacteria, promote plant growth under stressful conditions, like drought and heat, high salinity and poor nutrient availability. The molecular mechanisms for increasing stress tolerance in plants by endophytes include induction of plant stress genes expression, as well as biomolecules like reactive oxygen species (ROS) scavengers (Gałazka et al., 2015; Lata et al., 2018).

6.7 IAA and phytopathogens

Auxins biosynthesis is connected to the synthesis of various defence molecules, increased auxins production in infected plants leads to the induction of some defence-related genes of the JA-pathway, what causes the increase of the defence response against pathogens (Ludwig-Müller, 2015). Auxins are important factors in bacterial plant diseases resistance and their defence systems. The mode of action of this phenomenon is based on the fact that Aux-IAA repressor proteins are not decomposed and can repress auxin response genes. This means that plant defence genes are up-regulated and plants may use their means of defence against pathogen attack (Duca et al., 2014). On the other hand plant-pathogenic bacteria and fungi are able to control plants by manipulating auxins. The ability is a well-known feature for biotrophs and less for hemibiotrophs or necrotrophs. Auxins may induce many genes what leads to reconstruction of the cellular program in that way in which cell favors pathogens. Auxins biosynthesis is directly associated with the synthesis of different defence compounds including indole moiety. Plant cell rigidity may be regulated via pathogen induced auxins - the conjugation to aspartic acid by HG3.2 molecule leads to the activation of the pathogenicity genes in the pathogen and to higher plant susceptibility. Downregulation of the expression of the *GH3.2* gene leads to the higher plant resistance (Ludwig-Müller, 2015). Endophytes are able to induce plant resistance system called induced systemic resistance (ISR). The most frequently discovered bacteria able to induce ISR are those from genera *Pseudomonas* and *Bacillus*. They are well known producers of auxins, mainly IAA, what is regarded as a molecule increasing colonization efficiency by interfering with the host defence system (Hardoim et al., 2015). Induction of resistance by endophytes was described by many authors; they mentioned numerous bacteria beneficial in this process. But sometimes using endophytic bacteria leads to other beneficial features, e.g. *Azospirillum brasiliense*, which is able to elicit the resistance on antracnose in strawberry plants, cause increase of the tolerance on salinity and drought stresses (Gałązka et al., 2015). On the other hand, Wang et al. (2007) suggested that salicylic acid inhibits the auxin-signaling pathway, what indicates that plants able to accumulate salicylic acid are less sensitive to exogenously derived IAA, as a factor of defence system. This is the way of acting of pathogenic bacteria, which produce IAA and suppress pathogenic related gene (PR) expression, and in this way weaken salicylic acid dependent defence system (Duca et al., 2014).

Some of beneficial IAA producers are able to suppress phytopathogens development. *B. amyloliquefaciens* found on seeds of browntop millet (*Urochloa ramosa*) showed inhibition activity against fungal pathogens, including *Fusarium oxysporum*, *Curvularia* sp., *Alternaria* sp., and *Sclerotinia homoeocarpa* with percentage inhibition being 48.39%, 60.00%, 68.00% and 32.26%, respectively in antagonistic dual-organisms test on PDA medium (Verma and White, 2018). Improved resistance in sugar beet was observed by Nabrdalik et al. (2018) when sugar beet seedlings were infected by *Rhizoctonia solani* and simultaneously *P. agglomerans* BC17 as a bio-protectant. Plants inoculated only with bacteria showed lower dry mass content (although, not statistically significant). That result means that plants grown under the influence of *P. agglomerans* were more hydrated, and this may be the reason for their increased resistance. The mode of action of bacteria could be also due to their ability to reduce *R. solani* growth in cultures, to influence on the plant growth, and to produce IAA.

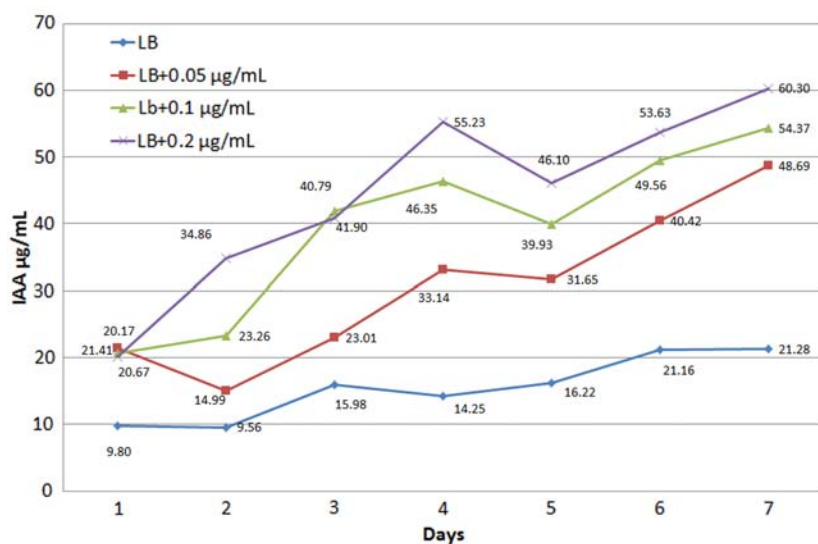
6.8 Applications

IAA is considered to be one of the most important factors in bacterially stimulated plant growth (Spaepen et al., 2007; Szkop et al., 2012). Paradoxically both, plant pathogens and PGP bacteria are able to produce IAA, so the first step in any application is to eliminate all possible pathogenic reactions. It is worth to remember also that some analogues of IAA like 2,4-D or NAA may increase plants susceptibility to diseases and promote phytopathogens growth (Spaepen et al., 2007).

Bacteria beneficial for plants (PGPB) may realize their defensive mechanisms towards plants protection including several modes of action as: competition for an ecological niche and for nutrients; production of antibiotics and enzymes lysing walls of plant pathogens; production of other secondary metabolites against pathogens; inducing systemic induction resistance (ISR) in host plants or inducing abiotic stresses (Pociejowska et al., 2014). One of the most important ways of action of PGPB is occupying a space around the roots. The spaces are rich in nutrients that attract lots of different microorganisms, including phytopathogens, and PGPBs protecting plants against phytopathogens by competing for those goods. IAA production is usually one of side effects which plays important role in the way to biological success. Beneficial usage of bacteria producing IAA is an alternative for chemicals in biocontrol and bio-stimulation of plants. Commercially applied strains are not numerous in Poland as well as in European Union, and usually they are not treated as IAA producers. Since now, in Poland, there have been registered only four bio-preparations based on *Bacillus* spp. and *Pseudomonas* sp. bacteria (Table 6.2). Those bacteria are probably potentially able to synthesize

TABLE 6.2 The bio-preparations containing potentially IAA producing bacteria and registered in Poland.

Bio-preparations	Microorganism	Strain [content in a preparation]	Year of registration/type of preparation
Amylo-X WG	<i>Bacillus amyloliquefaciens</i>	D747 [250 g/kg]	2018/fungicide
Integral Pro (also Serifel)	<i>Bacillus amyloliquefaciens</i>	MBI600 [6,8%]	2018/fungicide
Serenade ASO	<i>Bacillus subtilis</i>	QST 713 [13,96 g/L]	2015/fungicide
Proradix	<i>Pseudomonas</i> sp.	DSMZ 13134 [6,6 × 10 ⁸ jtk/g]	2018/fungicide

**FIGURE 6.1** The influence of tryptophan (0.05; 0.1 and 0.2 µg/mL) addition to LB medium on the ability of *B. subtilis* QST 713 (biofungicide Serenade ASO) to produce IAA (statistical analysis was done by ANOVA with differences evaluated by Tuckey test $p < 0.05$); test was prepared by the colorimetric method with the Salkowski's reagent with 30 mins. of incubation; absorbance at $\lambda = 530$ nm.

indole-3-acetic acid, but all of them are registered as fungicides. Proradix is a fungicide registered for protecting potato crops against *Rhizoctonia solani* and is based on naturally occurring bacteria, rather not endophytic ones. Serenade ASO is registered for protecting plants cultivated in the ground and under covers against fungal and bacterial diseases of pear, apricot, peach, cherry, plum, of gooseberry, chokeberry, highbush blueberry, blackberry, red currant, red, white, strawberry, cranberry, lettuce, tomato, eggplant, peppers cucumbers, carrots, parsnip, celery, cabbage, red cabbage, cauliflower, kale, broccoli, Chinese cabbage and Brussels cabbage. Our laboratory investigations showed that *B. subtilis* QST 713, the active ingredient of Serenade ASO, is able to produce IAA (Fig. 6.1), so its protective mode of action should be considered as a possibility to stimulate plants growth. *B. subtilis* QST 713 is able to synthesize 21 µg/mL of IAA in the absence of tryptophan after seven days of incubation, and over 60 µg/mL of IAA with the 0.2% addition of tryptophan to the medium (Fig. 6.1). The experiment carried out in our laboratory showed that the strain is tryptophan-independent of auxin production, and the amount of IAA grows proportionally to the tryptophan addition. Other two preparations, also registered in Poland as fungicides, contain *B. amyloliquefaciens*, strains MBI600 (Integral Pro) and D747 (Amylo-X WG). *B. amyloliquefaciens* MBI600 is also an active ingredient of Serifel – bio-fungicide registered in European Union. Although producers do not inform about their biochemical activity, including ability to produce IAA, it could be expected that they can synthesize IAA, and that this is at least one of their beneficial features. Because, it is well documented that bacteria produced IAA may stimulate hairy roots formation giving better development of primary and next lateral roots, the next consequence is increase of their number and length. The result of this is improved development of rooting system in soil, and a higher water and minerals uptake, what leads to exceed nutrition of plants and their better condition. This way of acting is reserved only for optimal doses of bacteria or bio-preparations, because in higher concentrations they may inhibit root system development (Duca et al., 2014; Pocijowska et al., 2014; Lata et al., 2018).

Tamreihaoa et al. (2016) discovered and described beneficial for plants *Streptomyces corchorusii* strain UCR3-16. They also prepared a formulation test with talc, as a carrier substance, for the strain. The strain was obtained from rice

rhizosphere soil and showed antifungal activities against major rice fungal phytopathogens. The mode of action of the strain was due to production antifungal cell wall degrading enzymes such as chitinase, β -1,3-glucanase, β -1,4-glucanase, lipase and protease. On the other hand, the strain also affected positively plant growth by producing up to 30.5 $\mu\text{g/mL}$ of IAA, siderophores, and solubilizing inorganic phosphates. It also produced ammonia and gave positive results for ACC deaminase activity. Rice plants infected by pathogen, and treated with talc powder, used as a bacteria carrier, showed increased root and shoot length, as well as fresh and dry root and shoot weight, and total grain yield, in greenhouse and field tests. In this context the strain of *S. corchorusii* seems to be a good active ingredient of a potential bio-protective preparation.

Typical ways of bacteria inoculum preparation involve two steps, first is the initial inoculum growing on the particular medium, the second – is mixing the bacterial suspension with talc, turf, peat moss or medium, resulting in solid or liquid preparations.

6.9 Conclusion

Many researches show evidences for IAA production by numerous endophytic bacteria originating from various plant species and from various plant organs and tissues. All of them are potentially good sources of active ingredients for bio-preparations used in agriculture for improving plant growth and development, and some of them may be also expected as good bio-protectants against phytopathogens. Huge number of bacteria able to auxins synthesis gives for researchers' great opportunity to find those which are the best; however, researches have to be realized very carefully and comprehensively, because of the possible presence of some pathogenic IAA producers. The border between promotional and pathogenic activity of IAA producing bacteria in some cases seems to be very thin, but distinct, nevertheless researches on them would be fruitful for both, agriculture and environment. This rises from enhancing seedlings' vigor via phytohormone production by beneficial bacteria, as well as increased tolerance to soil pathogens. And a growing demand of extending farming systems in extensive agriculture will use higher efficiency in nutritional uptake and better tolerance to environmental stresses under the influence of beneficial bacteria including those able to produce IAA.

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Chapter 7

Molecular aspects of biocontrol species of *Streptomyces* in agricultural crops

Vivek Sharma¹, Anu Sharma¹, Anudeep B. Malannavar³ and Richa Salwan²

¹University Centre for Research and Development, Chandigarh University, Sahibzada Ajit Singh Nagar, India, ²College of Horticulture and Forestry (Dr. Y. S. Parmar University of Horticulture & Forestry) Neri, Hamirpur, India, ³Department of Plant Pathology, CSK HPKV, Palampur, India

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7.1 Introduction

The green revolution resulted enormous agricultural productivity which is mainly achieved by the implementation of synthetic pesticides and fertilizers. The continuous and extensive usage of chemicals such as organophosphates, pyrethroids, or ganochlorines and carbamates (Sutthanont et al., 2010; Bayen, 2012; Reegan et al., 2016), led to several side detrimental effects and ecological discrepancy (Semmler et al., 2010; Schmahl et al., 2010; Abdel-Ghaffar et al., 2010; Saurav et al., 2013). However, in present circumstances, including side effects of pesticides to the ecosystem, development of resistance in the pathogens due to coevolution, created concern among consumers and growers also restricted their usage. Therefore, efforts for alternative measures, for increasing crop yields are required. In addition to genetic engineering of plants for disease resistance, which is time consuming and has concerns for their acceptability in public, demands novel practices which can circumvent the current limitations of agricultural production in a sustainable way (Rey and Dumas, 2017). The focus of researchers to natural enemies, appears to be a better and sustainable solution (Saurav et al., 2013). The competitive interactions mediated through the recruitment of soil microbes affect the fundamental equilibrium of terrestrial ecosystem by determining the community assembly and succession, and primary productivity (Kardol et al., 2006; Schnitzer et al., 2011; Fitzpatrick et al., 2018).

Plant beneficial microorganisms find tremendous importance in the pharma and agricultural industries. The success of these microorganisms is attributed to their inherent ability to produce plethora of bioactive metabolites and proteins (Andersen et al., 2018). Moreover, the natural antagonistic microorganisms are explored as a potential alternative approach which could minimize the use of chemicals in agriculture (Sunpapao et al., 2018). These host-associated microbial communities either in the rhizosphere or as endophytes play key roles in nutrient acquisition, development and immunity of host plant (Fitzpatrick et al., 2018). Although, mechanisms responsible for contributing host–microbiome interactions inn this context are complex and are poorly understood, yet substantial studies have been carried. Actinomycetes representing Gram positive bacteria, are one of the largest group of bacteria which has been explored for their plant beneficial attributes (Sharma and Salwan, 2018; Salwan and Sharma, 2018). Initially recommended in 1943 by Waksman and Henrici, *Streptomyces* constitute the largest genus of actinobacteria. *Streptomyces* comprises a

group of filamentous, non-motile, aerobic, catalase positive, non-acid-fast bacteria, Gram-positive that look like fungi (Flärdh and Buttner, 2009; Hasani et al., 2014). *Streptomyces* with characteristic earthy smell, cosmopolitan nature, constitute around 10% of total soil microbial flora (Janssen, 2006). They are either free-living or symbiotically associated with plants, fungi and animals or sometime as plant pathogens (Seipke et al., 2012; Rey and Dumas, 2017). Presently, approximately 1000 *Streptomyces* species have been characterized from soils and sediments, collected across the world from terrestrial as well as aquatic habitats (King et al., 1991). There are several unverified studies in the literature of *Streptomyces* species caused crops diseases (Loria et al., 1997) however only a few plant pathogenic species are found for virulence in agricultural crops such as potato (Manzer et al., 1977), sweet potato (Clark and Matthews, 1987) and on other taproot crops (Lambert, 1991). The common scab of potato and related diseases of other plants caused by *Streptomyces scabies* are well known. Mostly infecting underground plant parts, the initial symptom appears in the form of necrosis. Systemic plant infection is never reported although aerial portions of plants can be stunted or wilted if root infection is severe.

Besides that, a number of members belonging to *Streptomyces*, produce various bioactive metabolites having anti-bacterial (Schumacher et al., 2003; de Lima Procópio et al., 2012; Lee et al., 2014a, b), antifungal (Lam, 2006), antiviral (Ara et al., 2014), immunosuppression anticancer, and antioxidant activities (Tan et al., 2015). These bioactive secondary metabolites of *Streptomyces*, contribute to approximately 75% of commercial antibiotics for pharmaceutical industries (Pelaez, 2006; Lee et al., 2014b; Kashif et al., 2016). In agricultural fields, *Streptomyces* species/ strains are explored for their biocontrol role against different plant pathogenic fungi (Gonzalez-Franco and Robles-Hernandez, 2009; Law et al., 2017). Additionally, *Streptomyces* can either inhibit or promote nitrogen-fixing symbiotic relationship between plants and bacteria (Tokala et al., 2002). The role of *Streptomyces* species as endophytes, as plant pathogens (Coombs and Franco, 2003; Loria et al., 2006), as biocontrol agents (Weller et al., 2002), led to several excellent reviews (Loria et al., 2006; Schrey and Tarkka, 2008). *Streptomyces* with endophytic life style are capable of managing plant pathogens, largely due to their ability to secrete biological active secondary metabolites. For example, endophytic strain of actinomycetes, enhances plant growth and suppress *Pythium aphanidermatum*, infecting cucumbers plants (El-Tarabily et al., 2009). Similarly, an endophyte *Streptomyces* sp. S96 strain, decrease the wilt disease caused by *Fusarium* and *Magnaporthe oryzae* (Cao et al., 2005; Zeng et al., 2018). Moreover, the colonization followed by sporulation within the external cell layers of root in pea and other leguminous crops by *S. lydicus* WYEC108 strain, increases the root nodulation frequency and average nodule size by *Rhizobium* spp. and improve the potency of bacteroid within the nodules by increasing the nodule accumulation of Fe^{3+} and additional soil minerals (Tokala et al., 2002).

The contribution of nearly 10% of soil microbial flora by *Streptomyces* members revealed their integral role in soil microbial communities (Janssen, 2006; Schrey and Tarkka, 2008). The plant beneficial activities of *Streptomyces* are largely attributed to competition for nutrients and space, antibiotics and lytic enzymes production (Gonzalez-Franco and Robles-Hernandez, 2009; Boukaew and Prasertsan, 2014), plant growth and plant immunity modulation. The potential to colonize plant root surface and endure under different types of soil and environmental conditions are other essential pre-requisite responsible for the success of these microorganisms (Gonzalez-Franco and Robles-Hernandez, 2009; Ningthoujam et al., 2009). Therefore, keeping in view the importance of *Streptomyces* in agriculture, present book chapter highlights, its role as biocontrol agents or plant probiotic agent and explore its molecular aspects which are beneficial to the crop health, and productivity.

7.2 Biocontrol

The natural ability of *Streptomyces* species as prolific producers of bioactive metabolites, makes both the strains and their metabolites, an attractive biofungicides for the management of different plant diseases. For examples, *Streptomyces* sp. Di-944 (Fig. 7.1A) is found to suppress damping-off caused by *Rhizoctonia solani* in tomato transplants (Sabaratnam and Traquair, 1996; Sabaratnam and Traquair, 2002) whereas *S. roseolus* (Fig. 7.1A) is used as bio-control agent against aflatoxin B1 contamination (Caceres et al., 2018). Similarly, *S. yanglinensis* 3–10, significantly reduced the *A. flavus* mycelial growth, protect food and feedstuffs from aflatoxin contamination (Kong et al., 2010; Shakeel et al., 2018). In Japan, the first commercial metabolite blasticidin-S from *S. griseochromogenesi*, was antibiotic used for the management of rice blast (Fukunaga et al., 1955; Takeuchi et al., 1958; Tapadar and Jha, 2013). Thereafter, another antibiotic Kasugamycin (Fig. 7.1C), discovered in 1965 was isolated from *S. kasugaensis* (Umezawa et al., 1965). Presently, the application of blasticidin-S (Fig. 7.1B) is decreased and it is currently replaced with new fungicides with less toxicity (Copping and Duke, 2007). Kasugamycin is currently still marketed. Due to low mammalian toxicity of kasugamycin and no phytotoxicity toward most crops, it is still used to provide protection against rice blast (Yamaguchi, 1982; Copping and Duke, 2007). The commercial formulations of Kasugamycin in the

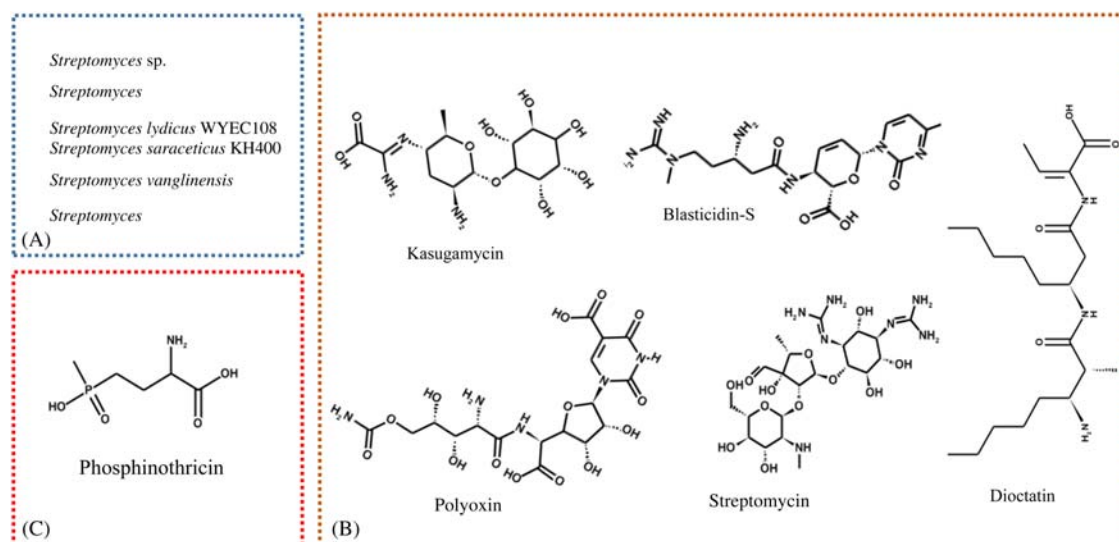


FIGURE 7.1 Examples of *Streptomyces* species and their bioactive secondary metabolites as biocontrol agents (A), biofungicides (B) and herbicidal agents (C) respectively.

form of wettable powder, granules, and liquids are available as Kasumin and Kasumin-Bordeaux (Copping and Duke, 2007; Hokko, 2015; Law et al., 2017). Moreover, the bioformulation derived from *Streptomyces griseoviridis* K61 available as wettable powder are used as biofungicide in greenhouse grown ornamental and vegetable crops against different soilborne pathogenic fungi, including *Alternaria*, *Botrytis*, *Phomopsis* and *Fusarium* (Tahvonen and Avikainen, 1987) and even against *Pythium*, *Phytophthora*, and *Rhizoctonia* (Tahvonen and Lahdenpera, 1988). Mycostop is also recommended by the Pest Management Regulatory Agency in Canada for the providing protection against damping-off, rot of root and stem and wilt of ornamental and vegetables crops caused by *Fusarium*. The *S. griseoviridis* strain K61 (Mycostop) is helpful in managing black scorch on date palm caused by *C. radicola* and soilborne pathogens such as *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium* of tomato (Suleman et al., 2002; Minuto et al., 2006).

In controlled conditions, a combination of zeolite and powdered milk based bioformulation of *Streptomyces violaceusniger* YCED9, is found effective against *Sclerotinia homeocarpa*, responsible for crown-foliar disease and *R. solani*, responsible for seedling disease in turfgrass (Trejo-Estrada et al., 1998a,b). Similarly, bioformulations of *S. hygroscopicus* strains YCED9 and WYE53, prepared in a zeolite carrier are found effective against Kentucky bluegrass seedlings against different fungal pathogens (Chamberlain and Crawford, 1999). Another bioformulation prepared from *Streptomyces* sp. 385 in zeolite carrier and amended with chitosan is used against *Fusarium* wilt in greenhouse-grown cucumber (Singh et al., 1999; Sabaratnam and Traquair, 2002). Due to production of antimicrobial bioactive metabolites and lytic enzymes such as chitinases and glucanases by *S. lydicus* WYEC108, *S. griseoviridis* K61, *S. violaceusniger* YCED9 and *S. saraceticus* KH400, these strains are recommended for the biocontrol of fungal and bacterial plant pathogens (Chater et al., 2010; El-Naggar et al., 2012; Kim et al., 2012; Lucas et al., 2013; Palaniyandi et al., 2013b; Nagpure et al., 2014; Kim et al., 2015; Klementz et al., 2015; Köberl et al., 2015; Van Minh et al., 2015). Bioformulations such as Actinovate® derived from *Streptomyces lydicus* WYEC 108 along with Mycostop are limited for soil treatments whereas purified metabolites kasugamycin, polyoxin D and streptomycin of *Streptomyces* are currently marketed as fungicidal and bactericidal as foliar spray (Rey and Dumas, 2017).

The commercial biocontrol strains of *Streptomyces* including *S. lydicus* WYEC108 and *S. saraceticus* KH400 (Elliott et al., 2009; Palaniyandi et al., 2013a,b) are comparatively safe for humans. However, in rare cases, hypersensitive response has been observed during exposure from dust, produced during handling of *S. griseoviridis* strain R K61 (Mycostop) as dry end-product (Pest Management Regulatory Agency, 2003). A large number of metabolites with herbicidal, activity have been identified from *Streptomyces* spp. which can be explored for the biocontrol of weeds (Bignell et al., 2010; Lucas et al., 2013; Klementz et al., 2015). For example, phosphinothricin (Fig. 7.1B), produced by *Streptomyces* is glutamine synthetase inhibitor has been used as herbicide in particular selection of transgenic plants (Schwartz et al., 2004; Zhang et al., 2014). However, the strains recommended for use in agricultural applications, needs to be tested for the production of any phytotoxic compounds, prior to its release. For example, strains deficient

for thaxtomin A, responsible for inhibiting the cellulase synthase may be explored for its biocontrol role (Lin et al., 2012; Rey and Dumas, 2017).

7.3 Mechanism of *Streptomyces* as plant beneficial microbes

Streptomyces species have been reported from extreme habitats including soil, plant parts and even from air (Kettleson et al., 2013; Supong et al., 2016). *Streptomyces* species are known to secrete thousands of bioactive metabolites. The extracts obtained from liquid cultures of *S. yanglinensis* isolate 3–10, suppresses the growth of several plant pathogens (Lyu et al., 2017). Additionally, these metabolites are source of anticancer molecules, vitamins, and antibiotics (Berdy, 2005; Gallagher et al., 2010; Manivasagan et al., 2014). In agriculture sector, potential *Streptomyces* species and their metabolites (Al-Bari et al., 2007; Zucchi et al., 2008; Lyu et al., 2017) or their enzymes including chitinases (Gomes et al., 2001; Mander et al., 2016) are explored for their antagonistic action against plant pathogenic fungi (Berg et al., 2001; Minuto et al., 2006; Law et al., 2017). Further, Blastidicin A and Diocatin A (Fig. 7.1C) (Yoshinari et al., 2007) obtained from *Streptomyces* is found to suppress the plant pathogens (Lyu et al., 2017). With a genome size of nearly 8–9 Mbp size and over more than 70% of GC content (Wu et al., 2005; Hasani et al., 2014; Ser et al., 2015c; Law et al., 2017). The draft genome annotation of *Streptomyces* sp. TOR3209 with 8 Mb genome size and GC content of over 72% encode 7564 proteins. Another hallmark feature of *Streptomyces* TOR3209 strain revealed that its genome contain genes, involved in the degradation of carbazole, naphthalene, anthracene, atrazine, ethylbenzene, fluorene, xylene, toluene, trinitrotoluene, and other toxic substances (Hu et al., 2012).

7.4 Secondary metabolites of *Streptomyces*

Among actinobacteria, the genus *Streptomyces* possess complex life cycle (Flärdh and Buttner, 2009) and produces several secondary metabolites of diverse nature and activity (Challis and Hopwood, 2003). These metabolites modulate the physiology of the organisms, and are involved as chemical messenger of organisms with their surrounding environment. In addition, different active secondary metabolites are commercially explored in medicine as anti-infective, immunosuppressant and antitumor drugs (Challis and Hopwood, 2003), in human, animal and even in plant diseases management (Firm and Jones, 2003; Salwan and Sharma, 2018). These bioactive compounds revolutionized the pharma industry in the 20th century and played vital role in the development of potential life-saving procedures for heart surgery, transplantation and other sectors (Seipke et al., 2012). *Streptomyces* sp. CS isolated from Chinese medicinal plant *Maytenus hookeri* produces polyketides and bafilomycin (Lu and Shen, 2003; Lu and Shen, 2005; Li et al., 2008; Li et al., 2010). From its extracts, naphthomycins A (Fig. 7.2), E, K, D, L, M and N have been characterized for their weak antifungal and other activities (Yang et al., 2012; Yang et al., 2018b).

Since, the initial discovery of *Streptomyces* for metabolites production, Waksman and his research group discovered streptomycin (Fig. 7.1B) from *S. griseus* which was effective antibiotic against *M. tuberculosis*

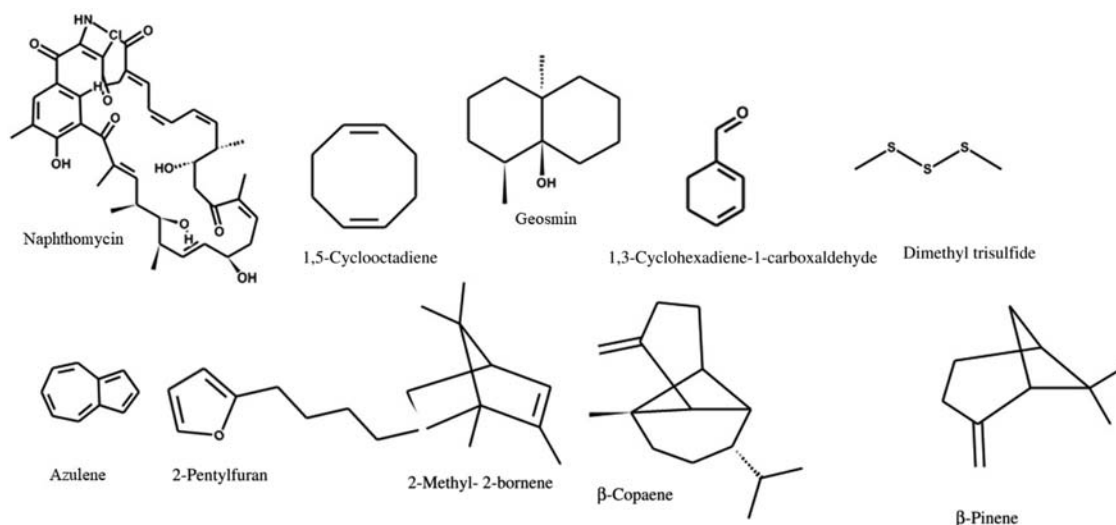


FIGURE 7.2 GC–MS profiling of volatile compounds produced by *Streptomyces* species with antifungal activity.

(Schatz and Waksman, 1944). Thereafter, another metabolite called geosmin (Fig. 7.2), responsible for characteristic 'earth smell' was discovered as indicator of abundance of these bacteria (Seipke et al., 2012). In terms of secondary metabolite production *Streptomyces* spp. have enormous hidden potential of secondary metabolite production and presently considered as workhorse organisms for biotechnological engineering to produce metabolites (Baltz, 2015; Cha et al., 2016). *Streptomyces* spp. are reported both as plant beneficial and detrimental microorganisms. So far, at least 20 metabolites have been characterized (Lucas et al., 2013; Klementz et al., 2015) for their insecticidal (Kaur and Manhas, 2013), nematocidal (Samac and Kinkel, 2001; Yang et al., 2013; Rashad et al., 2015), or molluscicidal activities (Chaosu et al., 1993; Lucas et al., 2013; Klementz et al., 2015; Rey and Dumas, 2017).

7.5 Volatile compounds

Biological solutions have emerged as a potential viable alternative in organic farming (Nguyen, et al., 2017). VOCs offer several advantage of being homogeneous due to their gaseous nature which can be been applied to foods protection without causing any organoleptic variations (Passone and Etcheverry, 2014). VOCs protect cereals from fungal outbreak and ensure protection with minimal or no organoleptic changes (Schotsmans et al., 2008). VOCs produced by microorganisms typically contains a mixture of several low molecular weight, diffusible compounds which can effortlessly evaporate at room temperature and pressure. Rhizobacteria in particular *Streptomyces* produce VOCs are found to exhibit antifungal activity (Wan et al., 2008; Li et al., 2010; Citron et al., 2015; Cordovez et al., 2015; Schmidt et al., 2015; Hung et al., 2015).

Volatiles secreted by *S. platensis* strain F-1, led to the development of resistance in rice against *Rhizoctonia solani*, in oilseed rape and strawberries plants against *Sclerotinia sclerotiorum* and *Botrytis cinerea* respectively (Wan et al., 2008). VOCs emitted by *S. albobflavus* TD-1 from China possess activity against numerous fungal pathogens (Wang et al., 2013a; Wang et al., 2013b; Yang et al., 2018a). The VOCs produced by 8 days old culture of *S. albobflavus* TD-1 grown on wheat bran are found to suppress the *A. ochraceus* growth (Yang et al., 2018a). VOCs produced by *S. albobflavus* TD-1 are also found to inhibit the mycelial growth *Fusarium moniliforme*, *Aspergillus flavus*, *Penicillium citrinum* and even inhibit the growth of gram- positive bacteria, due to the production of cyclic hexapeptides. The GC–MS analysis of the VOCs revealed 35 different compounds, belonging to numerous categories including alkanes, alkenes, alcohols, aldehydes, ketones, aromatic hydrocarbons, esters, ethers, and furans. Among different compounds, 2-methylisoborneol was reported in abundance followed by 1H-Indene. Beside this, other compounds including 1,3-cyclohexadiene-1-carboxaldehyde, 2-methyl- 2-bornene, 2-pentylfuran, 1,5-cyclooctadiene, bicycle non-3-ene, azulene, 4a (2 H)- naphthalenol (geosmin), and β -copaene are also reported with moderate abundance (Fig. 7.2) (Yang et al., 2018a).

Dimethyl trisulfide was found to be most effective and totally suppressed the mycelial growth of *A. ochraceus* at 10 μ L/L concentration level whereas benzenamine entirely inhibited the mycelial growth at higher concentration at 1000 μ L/L. On the other hand, metabolites such as anisole, β -pinene and 1,5-cyclooctadiene showed moderate activity against *A. ochraceus* 1000 μ L/L concentration (Yang et al., 2018a). The VOCs of several *Streptomyces* strains isolated from the rhizosphere of sugarbeet seedlings in disease-suppressive soils, promoted growth in *Arabidopsis* plants. The cascade of event involved in perceiving VOCs by *Arabidopsis* are still unknown, and it may be due to the association of cytokinin signaling pathway (Ryu et al., 2003).

7.6 Antibiotics

Streptomyces among actinomyces are the prominent producers of secondary metabolites (Lucas et al., 2013; Mahajan and Balachandran, 2014; Zhen et al., 2015). In agricultural sector, bioactive metabolites of *Streptomyces* are explored an alternate to fungicides for the managing phytopathogens plant diseases, an alternative to synthetic fungicides (Prabavathy et al., 2006). These biologically synthesized compounds are often biodegradable and may be useful for the developing pathogen-specific agricultural fungicides with minimum side- effects toward the ecosystem (Varma and Dubey, 1999; Prabavathy et al., 2006). Peptide antibiotics producing *Streptomyces* sp. (Zhao et al., 2018) are used in a wide range of applications in pharmaceuticals and agricultural sectors (Donald and Selman, 1948; Khalil et al., 2014; Zambry et al., 2017). For example, cyclic lipopeptide daptomycin obtained from *S. roseosporus*, is the first FDA-approved is one of the most important antibiotics in the past 50 years (Sauermann et al., 2008; Liu et al., 2018). In agriculture, antibiotics derived from *Streptomyces* spp. such as geldanamycin and nigericin are used for the management of plant pathogens (Fig. 7.3) (Rothrock and Gottlieb, 1984; Trejo-Estrada et al., 1998a,b). Oligomycin A of *S. diastatochromogenes* inhibit other phytopathogenic fungi including *Alternaria alternata*, and *Aspergillus niger*, *Cladosporium*

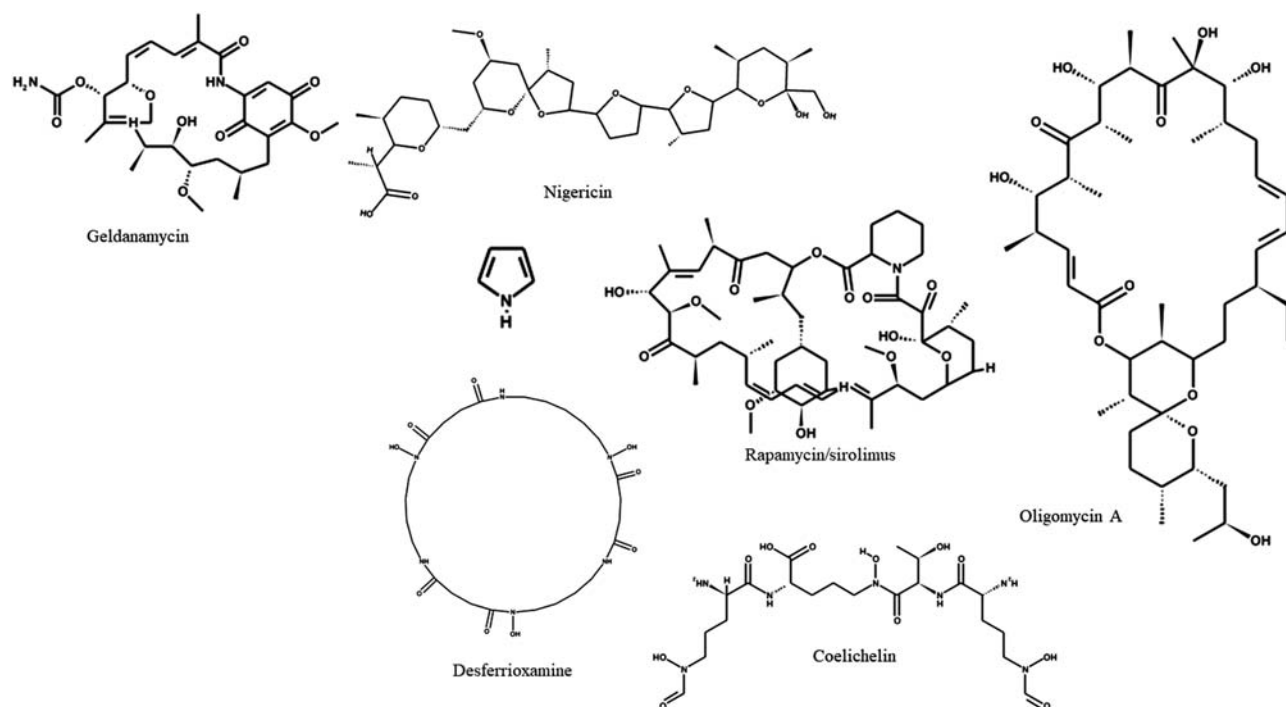


FIGURE 7.3 Examples of antibiotics and siderophores produced by *Streptomyces* and used for the control management of plant pathogens.

cucumerinum, *M. oryzae*, *B. cinerea*, *Colletotrichum lagenarium*, *Phytophthora capsici* (Kim et al., 1999; Yang et al., 2010). Oligomycin A treated rice plants at 50 $\mu\text{g/mL}$ led to reduction in lesions and upon increasing the concentration upto 500 $\mu\text{g/mL}$, the complete alleviation of symptoms was observed (Kim et al., 1999). Other antibiotic, “Rapamycin” also known as “Sirolimus” of *S. hygroscopicus* was found against *Candida albicans*, and other fungi (Sehgal et al., 1975). Rapamycin also inhibit the *Mucor circinelloides* and *Rhizopus oryzae*, responsible of post-harvested diseases in fruits (Johnson et al., 1990; Kwon et al., 2011). Other antibiotics derived from pyrroles and its derivatives from *Streptomyces* species, provide protection in plants against phytopathogenic fungi (Robertson and Stevens, 2014; Ser et al., 2015b; Tan et al., 2015; Awla et al., 2016).

7.7 Iron acquisitions

Iron being one of the essential element is vital for various primary metabolic processes such as photosynthesis, respiration, and DNA replication etc. across the different biological entities (Schaible and Kaufmann, 2004). However, the poor solubility of iron in aerobic environments in the form of ferric iron (Fe^{3+}), it is not available for organisms (Haas et al., 2008). Consequently, organisms particularly microorganisms, utilize different tools to sequester soluble form of iron from the surrounding environment. A common approach, involved the biosynthesis and secretion of metal chelating class of organic compounds known as siderophores which are having high affinity for Fe^{3+} (ferric iron). Siderophores are produced by microorganisms to overcome low iron availability. They differ in their structure and ability to sequester iron. They are synthesized either through non-ribosomal peptide synthetase (NRPS) or NRPS-independent pathway (Walsh and Marshall, 2004; Oves-Costales et al., 2009). The secreted siderophores bind Fe^{3+} and the ferric complexes upon interaction with receptors, enter the cell where ultimately Fe^{3+} dissociate from the siderophore for use (Crosa, 1989).

The affinity of bacterial siderophores, compared to fungal siderophores for iron is high and play vital role in modulating the microbial communities in soil (Zeng et al., 2018). Three structurally different types of hydroxamate siderophore, (DFOE, DFOB, and Cch) have been reported from *Streptomyces acidiscabies* E13 strain which promote the cowpea plants growth in absence of both iron and nickel (Dimkpa et al., 2008). In another study, siderophore producing, phosphate solubilizing and auxin producing isolate of *Streptomyces* responsible for promoting plant growth under saline soil conditions has been reported. *Streptomyces*, mostly produce trihydroxamate type of siderophore such as desferrioxamine (Fig. 7.3) (Imbert et al., 1995; Yamanaka et al., 2005) but literature on enterobactin type of siderophore production

is also available (Fiedler et al., 2001). Reports on siderophore of coelichelin (Fig. 7.3) (Challis and Ravel, 2000) and griseobactin (Patzner and Braun, 2010) are also available (Lee et al., 2012). The antagonistic activity endophytic OsiSh-2 strain of *S. sporocinereus* against *M. oryzae* is probably linked to siderophore. Genomic annotation of *Streptomyces* also revealed high number of biosynthetic gene clusters for siderophore production among different actinomycetes (Zeng et al., 2018).

7.8 Lytic enzymes

The lytic enzymes with potential to target the fungal and bacterial cell wall and membrane associated proteins are implicated in the biocontrol of plant pathogens. These lytic enzymes include chitinases, glucanases, cellulases, proteases, and lipases. The lytic enzymes along with phytases, play vital role in maintaining soil fertility and health. The production of lytic enzymes under soil is influenced by organic matter composition as well as population structure of macro- and micro-organisms of soil. The genus *Streptomyces* among actinobacteria is extensively explored for cell wall-degrading enzymes (CWDEs) production (Chater et al., 2010). Chitinases and glucanases of *Streptomyces* spp. are capable of destructing fungal cell wall (Mahadevan and Crawford, 1996; El-Tarabily, 2003; Gonzalez-Franco and Robles-Hernandez, 2009). Chitinases cleave the β -1,4 glycosidic bonds between N-acetyl glucosamine units in fungal cell wall. For example, anthracnose management in pepper using biocontrol is correlated to the secretion of chitinases and glucanases by *S. cavourensis* SY224 strain (Lee et al., 2012). For example, the chitinase-secreting *S. violaceusniger* XL-2 inhibit the wood-rotting fungi (Shekhar et al., 2006). Moreover, the ability to produce lytic enzymes have diverse applications in a wide range of other applications such as food (Chen et al., 2010), and as antifungal agent (Wang et al., 2006; Gao et al., 2018). So far, several chitinases have been isolated from *Streptomyces* sp., including *S. roseolus* (Jiang et al., 2012), *S. anulatus* CS242 (Mander, et al., 2016), *S. coelicolor* A3(2) (Nguyen-Thi and Doucet, 2016), *S. griseus* (Berger and Reynold, 1958), and *S. glauciniger* WICC-A03 (Awad et al., 2014). However, informations on cloning and expression are limited to a few species only such as *S. albolongus* ATCC 27414 (Gao et al., 2018). A list of selected fungal CWDEs along with metabolites responsible for antimicrobial of *Streptomyces* is described in Table 7.1.

7.9 Plant growth promotion and plant growth regulators production

A number of plant growth promoting rhizobacteria (PGPR) including *Pseudomonas*, *Bacillus*, *Azospirillum* and several affiliates of the genus *Streptomyces* are capable of plant growth promotion (Lehr et al., 2007; Schrey and Tarkka, 2008; Viaene et al., 2016a,b) and increasing crop yield by improving the soil environment either through the nutrient acquisition and through plant growth regulators secretion (Kruasuwan and Thamchaipenet, 2016). The inoculation of Arabidopsis, rice, wheat, sorghum and tomato plants with potential *Streptomyces* strains, enhances the plant biomass production (Dimkpa et al., 2008; Lin and Xu, 2013; Gopalakrishnan et al., 2013a,b; Jog et al., 2014; Palaniyandi et al., 2014; Cordovez et al., 2015). The other mechanisms used by the PGPR, to promote plant growth include nitrogen fixation, phosphate solubilization, aminocyclopropane-1-carboxylate deaminase (ACCD) production (El-Tarabily, 2008; Qin et al., 2014; Jaemsaeng et al., 2018), iron acquisition and sulfur oxidation (Bulgarelli et al., 2012). In rice and mung bean plants, the growth promotion by *Streptomyces* strain is associated with siderophore secretion (Rungin et al., 2012) (Fig. 7.4).

The biological properties and complete genome annotation of two antifungal and plant growth promoting strain; *S. albireticuli* MDJK11 (8.14 Mb) and *S. alboflavus* MDJK44 (9.62 Mb) isolated from the rhizosphere soil of peony in Shandong, China depicted protein-coding genes of 6550 and 7285 respectively. Additionally, two plasmids were reported in the MDJK44 strain (Wang et al., 2018). Among total predicted protein encoding genes, genes assigned to the COG databases for both strains MDJK11 and MDJK44 include 3137 and 3966 respectively. Both of these strains also produced siderophore on CAS-ager plates. The prediction for gene clusters using antiSMASH (v.4.1.0) revealed both nonribosomal peptide synthase (*nrps*) and polyketide synthase (*pks*) cluster of genes mainly involved in the biosynthesis of siderophore, terpenoid biosynthesis and other metabolites related to biological resistance. In MDJK11 genome, a total of 37 secondary metabolic gene clusters, responsible for 2-methylisoborneol (MIB) biosynthesis, teleocidin B biosynthesis, for ectoine biosynthesis and other metabolites has been reported. The MIB and teleocidin B types of terpenoids are synthesized by terpene synthases (Awakawa et al., 2014; Suurnäkki et al., 2015). Ectoine, an effective microbial osmo-stress protectant, could also serve as a versatile nutrient (Schulz et al., 2017). Furthermore, the other gene clusters involved in the biosynthesis of hopene, lantipeptide, cyclooctatin, albachelin, and candicidin are also

TABLE 7.1 Example of *Streptomyces* strains used explored for their role in agricultural applications.

S.No.	Strain	Lytic enzymes/compounds	Biological functions	Reference
1.	<i>Streptomyces</i> sp. NK10958	• Pironetin- a novel plant growth	Responsible for inhibiting the growth of rice plants without any loss of crop yield	(Kobayashi et al., 1994)
2.	<i>Streptomyces</i> sp.	• K10958P related to pironetin	NK10958P and pironetin responsible for stunted plant growth height dose of 109 and 115 ppm, respectively.	(Tsuchiya and Kobayashi, 1996)
3.	<i>Streptomyces</i> sp. GMKU 3100	• Siderophore-producing endophytic strain	Increased root and shoot biomass and lengths	(Rungin et al., 2012)
4.	<i>Streptomyces rochei</i> IDWR19, <i>Streptomyces carpinensis</i> IDWR53, <i>Streptomyces thermolilacinus</i> IDWR81	• Chitinases, phytase, alkaline protease, cellulase and invertase, • IAA and siderophore production	Plant growth promotion activity	(Jog et al., 2012)
5.	<i>Streptomyces</i> spp.	• Lipase, β-1–3-glucanase and chitinase	Antifungal activity against <i>Fusarium oxysporum</i> f. sp. <i>ciceri</i> and plant growth-promoting activity	(Gopalakrishnan et al., 2013a,b)
6.	<i>Streptomyces roseochromogenus</i> TSR12	• Chitinase genes	Antifungal activity against <i>F. oxysporum</i> f. sp. <i>cicero</i> , <i>R. solani</i> and <i>F. moniliforme</i>	(Debnath et al., 2013)
7.	<i>Streptomyces hydrogenans</i> DH16	• Indole acetic acid	Broad spectrum of antifungal activity against <i>Colletotrichum</i> spp., <i>Alternaria</i> spp., <i>Cercospora</i> sp., <i>Fusarium oxysporum</i> spp and plant growth promotion	(Kaur & Manhas, 2013)
8.	<i>Streptomyces glauciniger</i> WICC- A03	• Chitinase	Biocontrol of plant pathogens	(Awad et al., 2014)
9.	<i>Streptomyces exfoliatus</i> MT9	• Protease, chitinases, and β-1,3-glucanase	Biocontrol activity against <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	(Choudhary et al., 2014)
10.	<i>Streptomyces</i> mhcr0816	• Antifungal metabolites • Phosphate solubilization, phytase, siderophore and IAA production	Plant growth promoting and antifungal activity	(Jog et al., 2014)
11.	<i>Streptomyces</i> sp. Ach 505	• Chitinases • Provokes both ISR and SAR mediated plant immune responses	Resistance against oak powdery mildew	(Kurth et al., 2014)
12.	<i>Streptomyces violaceusniger</i> MTCC 3959	• Chitinase protease, and β-1,3-glucanase	Suppress phytopathogenic basidiomycetes	(Nagpure et al., 2014)
13.	<i>Streptomyces</i> sp. LuP30 and <i>Streptomyces</i> sp. LuP47B	—	Increased biomass and nodulation of <i>Medicago sativa</i>	(Le et al, 2015)
14.	<i>Streptomyces griseorubens</i> E44G	• Alkaline protease	Biological control of <i>Rhizoctonia</i> root rot disease of corn	(Al-askar & Rashad, 2015).
15.	<i>Streptomyces griseorubens</i> E44G	• Chitinase	Biocontrol of <i>Fusarium</i> wilt disease of tomato	Al-Askar et al. (2015)
16.	<i>Streptomyces</i> sp.	• Chitinase	Antifungal activity against phytopathogens	(Karthik et al., 2015)
17.	<i>Streptomyces</i> TM32	• Solubilize phosphate, siderophore and indole-3-acetic acid • chitinase	Biological control of <i>Rigidoporus</i> sp. causing white root disease in rubber tree	(Nakaew & Rangjaroen, 2015)
18.	<i>Streptomyces goshikiensis</i> YCXU	• Catalase, β-1,3-glucanase, chitinase and urease enzyme activities	Inhibited a broad range of phytopathogenic fungi	(Faheem et al., 2015)

(Continued)

TABLE 7.1 (Continued)

S.No.	Strain	Lytic enzymes/compounds	Biological functions	Reference
19.	<i>Streptomyces</i> spp.	• Volatile and non-volatile metabolites, siderophore, protease and chitinase production	Inhibition of wilt caused by <i>Fusarium oxysporum</i> f. sp. <i>ciceris</i> in chick pea	(Amini et al., 2016)
20.	<i>Streptomyces galilaeus</i> CFFSUR-B12	• Chitinases	Inhibition of <i>Mycosphaerella fijiensis</i> responsible for Black Sigatoka Disease of banana	(Castillo et al., 2016)
21.	<i>Streptomyces</i> sp. SNL2	• Hydrogen cyanide, indole-3-acetic acid and siderophores • Chitinase and β-1,3-glucanase activities, and inorganic phosphate solubilization	Reduced root rot and growth promotion of tomato seedlings	(Goudjal et al., 2016)
22.	<i>Streptomyces anulatus</i> CS242	• Chitinase	Antifungal activity towards fungal pathogen	(Mander et al., 2016)
23.	<i>Streptomyces hygroscopicus</i> B04	• Cell wall-degrading enzymes including chitinase, β-1,3-glucanase, cellulase and protease	Potential biocontrol agent for controlling strawberry root rot	(Shen et al., 2016)
24.	<i>Streptomyces corchorusii</i> strain UCR3-16	• Siderophore and IAA production • Solubilization of inorganic phosphate	Biocontrol and plant growth promoting activities	(Tamreihao, et al., 2016a,b)
25.	<i>Streptomyces aureofaciens</i> K20	• Amylase, protease, cellulase, chitinase, gelatinase and urease production	A potent antagonist against <i>Macrophomina phaseolina</i>	(Shrivastava et al., 2016)
26.	<i>Streptomyces albospinus</i> CT205	• Chitinase, β-glucanase • Heat-resistant antagonistic substance	Biocontrol potential against cucumber <i>Fusarium</i> wilt	(Wang et al., 2016)
27.	<i>Streptomyces</i> sp. MBRL 10	• Diffusible and volatile compound(s), • chitinase, β-1,3-glucanase, lipase, protease etc. • Hydroxamate type of siderophore	antagonism against fungal pathogens and growth promotion in rice plants	(Tamreihao et al., 2016a)
28.	<i>Streptomyces</i> sp. UPMRS4		Plant growth-promotion of rice and biocontrol efficacy against <i>Pyricularia oryzae</i>	(Awla et al., 2017)
29.	<i>Streptomyces cameroonensis</i> sp. nov.	• Geldanamycin and inorganic phosphate, production of siderophores and indole-3-acetic acid, and 1-aminocyclopropane-1-carboxylate deaminase activity	Strain was able to promote plant growth and protect against <i>Phytophthora megakarya</i> , the main causal agent of cocoa pod rot.	(Boudjeko et al., 2017)
30.	<i>Streptomyces griseus</i> S4-7	• wblE2 transcription factor	Involved in plant- protection and shows specific and pronounced antifungal activity against <i>Fusarium oxysporum</i> f. sp. <i>fragariae</i>	(Cho et al., 2017)
31.	<i>Streptomyces globosus</i> UAE1	• Antifungal metabolites such as volatile compounds, hydrocyanic acid and siderophores, • Chitinase and β-1,3-glucanase	Potential Effective Biocontrol Agent for Black Scorch Disease in Date Palm Plantations	(Saeed et al., 2017)
32.	<i>Streptomyces griseorubens</i> E44G	• Chitinase	Antifungal activity against the <i>F. oxysporum</i>	(Rashad et al., 2017)

(Continued)

TABLE 7.1 (Continued)

S.No.	Strain	Lytic enzymes/compounds	Biological functions	Reference
33.	<i>Streptomyces</i> spp.		Chickpea plant growth promotion, helper effect with <i>Mesorhizobium ciceri</i> and host-plant resistance induction against <i>Botrytis cinerea</i>	(Vijayabharathi et al., 2018a; Vijayabharathi, Gopalakrishnan, Kumar, et al., 2018b)
34.	<i>Streptomyces rubiginosus</i> SP24	Chitinase	Biological control of root-knot nematode infection in <i>Vigna radiata</i>	(Jha and Modi, 2018)
35.	<i>Streptomyces sampsonii</i>	Chitinases ChiKJ406136	Antifungal effect on four forest pathogens (<i>Cylindrocladium scoparium</i> Morgan, <i>Cryphonectria parasitica</i> , <i>Neofusicoccum parvum</i> and <i>Fusarium oxysporum</i>)	(Li et al., 2018)
36.	<i>Streptomyces</i> sp.		Biological control of <i>Eucalyptus robusta</i> Smith leaf blight, <i>Castanea mollissima</i> BL. blight, <i>Juglans regia</i> L. blight and root rot	(Vijayabharathi, Gopalakrishnan, Kumar, et al., 2018b)
37.	<i>Streptomyces</i> WZS1-1 and <i>Streptomyces</i> WZS2-1	Phosphate-solubilizing, siderophore IAA production	Plant growth-promotion and host-plant resistance inducers against <i>Botrytis cinerea</i> in chickpea Enhanced nodulation and nitrogenase activity in five chickpea genotypes	(Han et al., 2018)

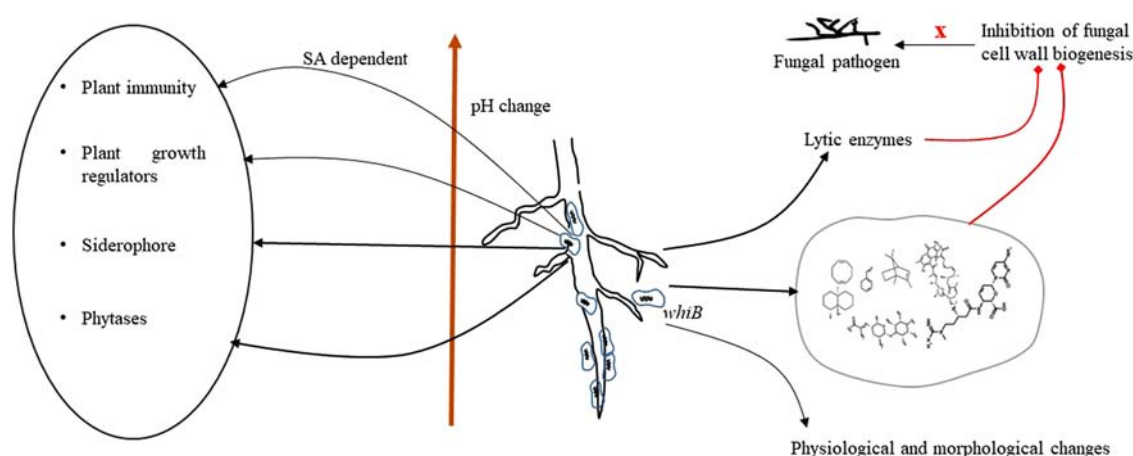


FIGURE 7.4 Effect of *Streptomyces* strains on plant growth, pathogen suppression and plant immunity. The inoculation of a combination of *Streptomyces* leads to an increase in the pH of the medium for a very short period which ultimately leads to the alkalization of extracellular medium. The regulatory element *whiB* —of *Streptomyces* determines different physiological and morphological changes involved in the differentiation and maturation of spore as well secondary metabolites production which suppresses the pathogens. The production of plant growth regulators, siderophores and phytases, promotes plant growth and crop yield.

predicted in the genome. The aromatic heptane known as candicidin is identified for its antifungal activity (Szczepblewski et al., 2017). In the MDJK44 genome, a total of 29 metabolic gene clusters are predicted in which the clusters for ectoine and albaflavenone biosynthesis are also predicted (Yamada et al., 2014; Gao et al., 2018) (Fig. 7.4).

The inoculation of *Ocimum sanctum* and tomato plants with ACCD- production, led to resistant under water logged conditions (Barnawal et al., 2012; Jaemsaeng et al., 2018). ACCD-producing microorganisms. increases the plant growth against both biotic and abiotic stresses, (Glick, 2004). *Streptomyces* sp. GMKU 336 can enhance salt tolerance in rice plants by converting ethylene precursor into α -ketobutyrate and ammonia through the action of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and finally leads to the ethylene level reduction. The ACCD-deficient mutant of *Streptomyces* sp. GMKU 336 increased plant height and biomass, chlorophyll content and adventitious roots formation, in mung beans, than non-inoculated plants (Jaemsaeng et al., 2018). In a nut shell, *Streptomyces* strains, developed numerous mechanisms to encourage plant growth still their role in planta substantiation is essentially lacking (Jaemsaeng et al., 2018).

Other aspects of plant growth promotion include, growth regulators production. The production of indole acetic acid (IAA), a natural auxin is already well reported for several actinobacteria (El-Tarabily 2008; Khamna et al., 2010; Legault et al., 2011; Rungin et al., 2012; Sadeghi et al., 2012). The IAA production in *Streptomyces* is dependent on indole-3-acetamide pathway from L-tryptophan (Lin and Xu 2013). *S. rimosus*, *S. olivaceoviridis*, and *S. rochei* are described positive for auxins, gibberellins, and cytokinin-like chemicals production which augment the plant growth of wheat (Aldesuquy et al., 1998). Endophytic strains of *Streptomyces* isolated from *Azadirachta indica*, release high level of IAA and encouraged the growth of tomato plants. Similarly, IAA production by another *Streptomyces* strain En-1 as endophyte encouraged the Arabidopsis plant growth (Lin and Xu 2013). In other study from yam rhizosphere, a total of 28 of 29 IAA producing isolates were reported (Palaniyandi et al., 2013a) in which only 11 isolates promoted the Arabidopsis growth under in vitro conditions (Palaniyandi et al., 2013a). The probable reason for lack of growth promotion by other isolates may be low concentration of IAA, existence of other chemicals which suppress the effect of IAA or additional requirements for plant growth promotion. However, *S. scabies* responsible for scab in maximum tap root and tuber crops is demonstrated to linked with IAA production hence it may act as a virulence factor (Vereecke et al., 1997) (Fig. 7.4).

The production of cytokinin is also reported in *S. turgidiscabies* (Joshi and Loria, 2007). However, it is a pathogen responsible for leaf galls in tobacco. An endophytic *S. hygroscopicus* strain TP-A045 is reported for pteridic acids A and B production with auxin- like activity, accelerate the adventitious roots formation kidney beans hypocotyls (Hasegawa et al., 2006) whereas in rhododendron seedlings, endophytic *Streptomyces* sp. MBR-52 strain produces root-promoting hormones (Hasegawa et al., 2006) (Fig. 7.4). Stimulation of root hairs and lateral root formation and secretion of sugar are the other physiological special effects mediated by of IAA (Davies 2004). The sugars secreted by the plant roots play vital role in early microbial root colonization (Dakora and Phillips, 2002). Thereafter, the growth or disease progression are directed by additional genes which are either beneficial or harmful to the plants.

7.10 Plant immunity

Plant immunity is a complex process and basal defense system which protect the host plant to compete against intruders (Vatsa-Portugal et al., 2017). The induced responses can be local or systemic in nature. It consists of physical barriers and several signaling pathways, involved in the recognition of pathogens through signal transduction which respond defensively through the expression of several genes and their products (Andersen et al., 2018). Plant beneficial microorganisms and their derived elicitors known as “microbial-associated molecular patterns” (MAMPs), acts as signaling molecules to activate the innate immune response of plants (Jones and Dangl, 2006). Further, MAMPs activate second line of induce systemic resistance (ISR) against the pathogen. Here, the cascade of events involved are MAP kinase activation, ion fluxes, alkaline extracellular environment and the release of reactive oxygen species (Van Loon et al., 2008), which ultimately leads to various molecular and cellular responses such as accumulation of phenolic compounds, deposition of lignin and callose, and hypersensitive response (Jones and Dangl, 2006; Farace et al., 2015). The induced responses may represent structural defenses, such as thorns and trichomes, to the production of pathogen related proteins (PR) and toxic chemical compounds such as nicotine and terpenoids (Bezemer and Van Dam, 2005). The activation of signal transduction network of plant immunity comprises salicylic acid (SA), jasmonic acid (JA), and/or ethylene (E) (Pieterse et al., 2014) depending on pathosystems (Kunkel and Brooks, 2002) (Fig. 7.4).

Streptomyces sp. in grape and Arabidopsis plants induce both local and systemic resistance against pathogens (Conn et al., 2008; Loqman et al., 2009; Couillerot et al., 2014). In Arabidopsis, *Streptomyces* mediated resistance appears to be salicylic acid dependent but not on jasmonic/ethylene pathways (Conn et al., 2008). Owing to the antagonistic nature, *Streptomyces* exert a selective pressure on soil microbes, which may not always work in favor for plant benefit as immune response. An endophytic plant growth promoting *S. anulatus* S37 strain, isolated from *Vitis vinifera* rhizosphere, enhance the plant immunity against numerous pathogens such as *B. cinerea* (Loqman et al., 2009). In spite

of the importance of *Streptomyces* as biocontrol and plant growth promotion, the cellular and molecular factors underlying their interaction with plant are still largely unknown (Vatsa-Portugal et al., 2017). During infection with *B. cinerea* in grapevine, and *S. anulatus* S37, prime the defense responses and causes remarkable decrease in pathogen-induced cell death. The prior interaction of grapevine with *S. anulatus* S37, leads to the development of refractory responses in plants against *B. cinerea*, thus revealing the differential nature of the host plant towards beneficial and pathogenic microbes (Vatsa-Portugal et al., 2017). In some cases, it promotes the formation of symbiotic relationship between plant roots and microbes, such as promotion of hyphal elongation of symbiotic fungi or root nodulation in leguminous plants (Schrey and Tarkka, 2008).

Alkalization of extracellular environment is a powerful indicator, for investigating the quick responses, mediating MAMP-induced defense responses (Felix et al., 1998; Küpper et al., 2001). For example, inoculation of grapevine cells with plant beneficial *S. anulatus* S37 strain alone or with the pathogen *B. cinerea* alone, a combination of *S. anulatus* S37 with *B. cinerea*, leads to change in pH of the medium by 1.1 unit within 10 minutes. The change in pH turns out be for a very short period and transient in nature for both *S. anulatus* S37 alone or *B. cinerea* alone, and returned to basal pH level after 120 minutes. However, inoculation of plants with a combination of *S. anulatus* S37 with *B. cinerea*, the alkalization of media was upheld for 150 minutes (Vatsa-Portugal et al., 2017). *Streptomyces* species also found to elicit systemic defense responses in oak plants upon pathogen challenge (Kurth et al., 2014). The regulatory element of *Streptomyces* origin such as whiB-like (wbl), named after the initial discovery in *Streptomyces coelicolor* (Rybniiker et al., 2010; Smith et al., 2010), determines different physiological and morphological alterations in actinomycetes (Cho et al., 2017). These elements are also involved in the differentiation and maturation of spores in streptomycetes (Chater and Horinouchi, 2003; Elliot and Talbot, 2004; Bibb, 2005), and other regulatory roles during developmental (Crack et al., 2009). These proteins are 10–15 kDa in size and consists of conserved cysteine residues which interact to iron–sulfur cluster (Jakimowicz et al., 2005). In *Streptomyces griseus* S4-7 strain, these element produces bioactive metabolites which inhibit fungal cell wall biogenesis, hence results the pathogen suppression. The genome sequencing and annotation identified several key genes related to secondary metabolite biosynthetic genes and diversity of regulatory DNA elements (Cha et al., 2016) (Fig. 7.4).

7.11 Conclusion

Stresses to the plants in both natural and managed ecosystems, adversely affects the plant growth and productivity. Microorganisms play vital role in combating plant stresses of both biotic and abiotic types (Fitzpatrick et al., 2018). *Streptomyces*, gained special attention from researchers, so far eleven commercial products have been approved in eight countries which clearly demonstrates that potential of *Streptomyces* metabolites in agriculture in compliance with regulatory processes across the globe. Recent developments in genomics, transcriptomics and morphological approaches, played vital role in understanding the complexity of its mechanism and further revolutionized the potential of *Streptomyces*. The fungicidal strains of *Streptomyces* for their action depend on the synergistic effects of different metabolites and extracellular enzymes (Rey and Dumas, 2017). The comparative genomic and transcriptomic level studies, already provided a deep insight about the number of secondary metabolite gene clusters encoded by PKS and NRPS in closely related *Streptomyces* strains and how a major share of them is silent in laboratory conditions (Rey and Dumas, 2017). The discovery of non-ribosomal peptide synthetase and polyketide synthetase biosynthetic gene clusters in *Streptomyces* genome and their modulation for secondary metabolites production, offers unique opportunities to engineer improved strains for agricultural applications.

Still, the positive role of *Streptomyces* on the plant health and productivity in Arabidopsis, alfalfa, sorghum, and rice plants and further in-depth analysis of their interaction will help in revealing the role of molecular arsenal of potential *Streptomyces*, on the plant phenotype (Xiao et al., 2002; Gopalakrishnan et al., 2013a,b; Palaniyandi et al., 2014). The developments in automated phenotypic screening assays, detailed monitoring of plant growth parameters, using kinematic analysis of leaf and root development will facilitate and promote the commercialization of the products (Klukas et al., 2014). However, due to the differences in environmental conditions across geographic locations, the impact of environmental parameters such as pH, organic matter, nutrients and soil moisture needs to be assessed and considered during the selection of potential biocontrol agents (Law et al., 2017). Besides the role of *Streptomyces* strains in inducing resistance against pathogens, promotion of plant growth, sincere efforts needs to be taken care, to avoid the selection of any virulent strain (Schrey and Tarkka, 2008).

The efforts to mine the wealth of possible *Streptomyces* for agricultural applications and parallel assessment of their limitations for translation of potential strains to effective products development with ethical and regulatory concerns is required. Moreover, there is need to unravel the potentials of *Streptomyces* using the synthetic biology and gene/genome

editing tools such as CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) for multiple modifications at genome level to augment, renovate, or quash the secondary metabolites clusters of genes which are useful and offers immense potential for developing environment friendly and economically viable alternate for agriculture over synthetic chemicals (Rey and Dumas, 2017). Additionally, the process optimization, use of novel economical carriers for bioformulation development, preventing the biosynthesis of deleterious metabolites and genetic engineering of the strains will help in industrial scale product developments for agricultural applications. In a nut shell, taken together, *Streptomyces* spp provide an alternative proof of concept and offers immense agricultural potential still the risk assessment of these strains needs to be addressed before the commercialization of the promising strain for agricultural sectors.

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Chapter 8

Root exudates, a key factor in the plant-bacteria interaction mechanisms

Oscar González-López¹ and David Ruano-Rosa²

¹Research Group in Engineering and Sustainable Agriculture, Department of Engineering and Agricultural Sciences, Environment Institute Natural Resources and Biodiversity, University of León, León, Spain, ²Woody and Horticultural Crops Unit (Agricultural Research Area), Technological Agricultural Institute of Castilla y León, Valladolid, Spain

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8.1 Introduction

Soil is a fascinating ecosystems in which a large range of microorganisms such as bacteria, actinomycetes, fungi, algae, protozoa and viruses live, establishing a complex network of interactions between them (Tiedje and Dazzo, 1982). These microorganisms, at the same time interact with other organisms, highlighting the relationship with plants through their roots. This association is related with a number of important ecological functions like: nutrient cycling, plant productivity, soil carbon sequestration, maintenance of soil structure, stress tolerance, biocontrol, bioremediation, biomass production and decomposition (Sharma and Verma, 2018). Current analytical techniques allowing functional genomics strategies such as metagenomics, metabolomics and metatranscriptomics have revealed the deep complexity of these interactions to the extent of being described by scientist as a meta-organism or holobiont instead of isolated individuals (Gupta et al., 2018).

The area of influence of plant roots, the so-called rhizosphere, is classified by many as the most crucial and active zone in the plant (Kennedy and de Luna, 2005; Dubey et al., 2017). In the rhizosphere there is a high microbial concentration, estimated for bacteria to be around 10^{10} per gram of soil, and a high diversity of taxa (Vacheron et al., 2013). This richness is closely related with root exudates (Badri and Vivanco, 2009). These compounds play an important role in the soil dynamics, since they are an important source of the organic carbon released by plant in the soil as part of the rhizodepositions (Jones et al., 2009; Pausch and Kuzyakov, 2018) and the principal reason why the number of bacteria around the roots is between 10 and 1000 greater than in the bulk soil (Choudhary et al., 2018). Consequently, root exudates are essential in plant-microbe communication with a proved capacity in shaping the root microbiome (rhizobiome) (Haichar et al., 2008; Kawasaki et al., 2016), attracting beneficial microbes and also rejecting the pathogens (Lundberg and Teixeira, 2018). But this selecting effect has been proved to be smaller than those caused by environmental or edaphic characteristics (Chen et al., 2005; Cúcio et al., 2016; Poudel et al., 2019). Additionally, for others authors, even considering root exudates as an important player in the rhizosphere, it is not clear that they establish the overall structure of rhizosphere bacterial communities (Dennis et al., 2010). Therefore, the study of the microbial communities is essential to elucidate the modulation capacity of these exudates. In this direction, constant evolution on sequencing technologies is improving the output capacity of the sequencers (Heather and Chain, 2016; Rascovan et al., 2016; Mitsuhashi et al., 2017), saving cost, time and allowing the access to a cheaper and better defined microbiomes.

Oburger and Jones (2018) define root exudates as plant-derived primary and secondary metabolites of both low molecular weight (MW) (<1000 Da; e.g. sugars such as fructose, glucose or maltose; organic acids such as malic acid, ascorbic acid or acetic acid; phenolics and vitamins) and high MW compounds (> 1000 Da; e.g. enzymes, mucilage). Exudates composition and production vary depending on biotic (e.g. plant species, developmental stage, genotype or even anthropogenic influence) and abiotic factors (e.g. environmental factors, soil compaction, water and nutrient availability, pH) (Badri and Vivanco, 2009; Lakshmanan et al., 2014; Haldar and Sengupta, 2015; Eisenhauer et al., 2017; Ghosh and Ray, 2017). There is a complete set of mechanisms involved in the exudation of these compounds, that can be divided in passive or active depending on the release procedure. When the exudation is a consequence of the diffusion of molecules through the plasma membrane as part of the concentration gradient cytoplasm-soil, the mechanisms are defined as passive, while the active mechanisms are those where the release is performed by the secretion through protein transporter channels and exocytosis of vesicles (Oburger and Jones, 2018). Passive mechanisms are used by the most of the low molecular weight organic compounds and, on the other hand, active mechanisms are used to release compounds of higher molecular weight (Huang et al., 2014). An exhaustive list of metabolites together with the root transporter families (for both uptake and release) can be consulted, for instance, in Sasse et al. (2018).

These phytochemicals take part in numerous biological interactions such as plant-plant (Peer and Murphy, 2006; Subrahmaniam et al., 2018), plant-fauna (Johnson and Gregory, 2006; Hiltbold et al., 2013), and naturally, plant-microorganism (Doornbos et al., 2012; Singh et al., 2017) (Fig. 8.1). Those interactions can be classified as either positive, such as mycorrhizal fungi or plant growth promoting rhizobacteria (PGPR), or negative, like parasitism or competition due to plant pathogens or parasitic plants (Haldar and Sengupta, 2015). Root exudates help in the set-up of the rhizospheric microbiome, which is implicated in plant defense against phytopathogens and/or growth promotion

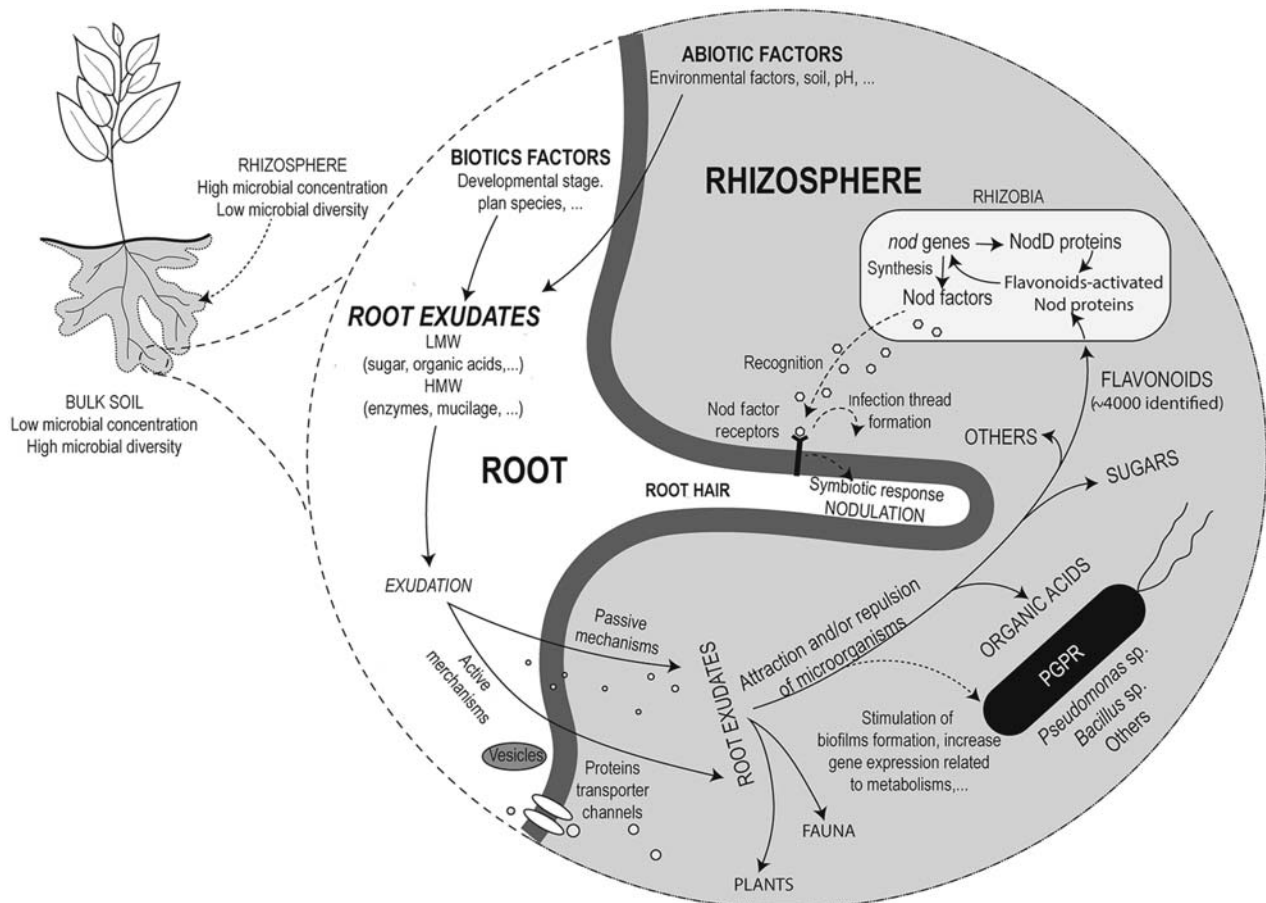


FIGURE 8.1 Schematic overview of complex interactions involving the root exudates emphasizing the relationship with bacteria. Root exudates are involved in the attraction and repulsion of different macro/microorganisms. These results, in the case of bacteria, in a high microbial concentration in the rhizosphere with an impoverishment of diversity if comparing with the rest of soil. The nature of root exudates can be very varied, highlighting for instance the flavonoids, due to role in rhizobia-legume interaction, or others like some organic acids described in the interaction with PGPR.

(Dubey et al., 2017; Mavrodi et al., 2017). Since this revision is focused on a particular group of microorganisms, the bacteria, and only on those involved in positive interactions, interested readers in other microorganisms such as fungi, or other kind of interaction are encouraged to consult, for instance, Lugtenberg (2016), Hakeem and Akhtar (2016) or Singh and Singh (2018).

The research on bacterial-root exudates molecular interaction has been faced both *in vitro* and *in planta* using different strategies like microarray analyses, promoter trapping, cDNA-based suppression-subtractive hybridization, gene silencing, mutagenesis, FISH or omics new approaches, assisted by novel techniques and bioinformatics (Barea, 2015).

But which are the benefits of knowing these interaction networks and their molecular characteristics? To briefly answer this question, which will be extensively addressed at the end of this chapter, we can take as example the legume *Medicago truncatula*. Under low nitrogen availability, the plant increases the expression of genes related with the generation of specific flavonoids involved in attracting and favor the symbiotic relationships with the rhizobia bacteria *Sinorhizobium meliloti* (Zhang et al., 2009). Consequently, we can improve the efficiency of this or other crops if we know in which point of this complex network is possible to introduce changes in our own interest.

The aim of the present chapter is to provide a brief overview on the research efforts focused to a better understanding of plant-bacteria interactions. More specifically, we focus on the implications that root exudates have in this interactions network, analyzed from a molecular point of view. Finally, we briefly discuss how the better knowledge of this subject will contribute to improve plant crops.

8.1.1 Root exudates and nitrogen-fixing bacteria

In the rhizosphere, a complex biological scenario where multiple interactions are being carried out, root exudates prove not to be only a supporting actor but an important co-star. Even when produced in small amounts, exudates are involved in several rhizosphere processes (Oburger and Jones, 2018). The study of the molecular aspects of these interactions had been mainly addressed on herbaceous plants. Legumes, because of the importance of atmospheric nitrogen fixing bacteria, rice, maize, wheat, or *Arabidopsis thaliana* (Riely et al., 2004; Barret et al., 2009; Chaparro et al., 2013; Xie et al., 2015; Mwita et al., 2016) as a model plant are some of the most used plants. However, to the best of our knowledge and as in other research areas (Ruano-Rosa and Mercado-Blanco, 2015), little is published regarding woody plants, other than seeds.

As above mentioned, one of the most studied cases in plant-microbe signaling is the root nodules formation for nitrogen fixation. This mutualistic relationship, in which the bacteria take nutrients from the host plant in exchange for nitrogen, is specialized for numerous plant-rhizobium pairs (Perret et al., 2000; Bais et al., 2006; Liu and Murray, 2016). In order to establish this relationship, plants attract compatible bacteria species and/or repel the undesirable ones through specific flavonoids released in the root exudates (Peer and Murphy, 2006). Flavonoids are a large group of plant secondary metabolites (~4000 identified) derived from the phenylpropanoid pathway (Abdel-Lateif et al., 2012) required by bacteria to initialize the transcription of *nod* genes. A complete list of flavonoids implicated in the attraction of rhizobia, and released by different legumes in their root exudates is depicted by Haldar and Sengupta (2015). *Nod* genes are accountable of the Nod factors synthesis. To date, have been identified several *nod* genes in *Rhizobium*: *nodA*, *nodB*, *nodC*, *nodD*, *nodE*, *nodF*, *nodI*, *nodJ*, etc. Detailed information about *nod* genes functions is described in Garcha and Maan (2017). Nod factors are signaling molecules (product of *nodABC* genes expression) released by rhizobia during the first stage of nodulation and chemically defined as lipochitooligosaccharides (Denarie et al., 1996). *NodD* gene is the transcriptional regulator of common *nod* genes expressed in the presence of certain flavonoids (e.g. naringenins) (Hu et al., 2000). But nodulation in legumes-rhizobia system, is a sophisticated process that involves the participation of other factors than flavonoids and Nod factors, and dependent of several genes (i.e. *nol* or *noe* genes) (Laranjo et al., 2014). The molecular complexity of this dialogue can be illustrated by some examples. Zhang et al. (2009) by silencing chalcone synthase (an important enzyme involved in flavonoids structure synthesis) achieved transgenic plants of *M. truncatula* deficient in the biosynthesis of flavonoids. Additionally, plants with isoflavone synthase and flavone synthase silenced genes resulted in roots with lack of two kinds of flavonoids *viz.* isoflavonoids (formononetin and biochanin A) and flavones (7,4'-dihydroxyflavone) respectively. As a consequence, using the rhizobium bacteria *S. meliloti*, practically absence of nodulation was obtained in plants with chalcone synthase silenced and also, a reduction in nodulation was observed in the flavone-deficient roots. In isoflavone-deficient roots the nodulation was normal. In the same study it was observed, the importance of flavones as internal inducer of *nod* genes and the important function of the flavonols as auxin transport regulators during nodulation. Likewise, Subramanian et al. (2006) approached the essential importance of isoflavones during nodulation process on soybean-*Bradyrhizobium japonicum*. For this purposed the isoflavone synthase expression was silenced by the application of RNA interference. As a result,

nodulation in roots fell down. This event was not reverted by the pre-treatment or exogenous application using the main soybean isoflavones. When the silencing treatment was focused on chalcone reductase, involved in the synthesis of daidzein, the genistein production was increased and no effects were observed on nodulation. All these experiments support genetically the initial hypothesis about the importance of isoflavones in soybean.

Rhizobia strains are host-specific, and not all leguminous plant species can establish a symbiotic relationship for nitrogen fixation. The level of this specificity may make impossible the colonization of the roots by other bacteria strains or even, after a satisfactory colonization developed nodules may not have the capacity of nitrogen fixation (Wang et al., 2018). This specificity can be due to both rhizobia and plant through flavonoids and flavonoid-NodD recognition, Nod factor recognition, surface polysaccharides and secreted proteins, the own host immunity or in the last stage, during the nitrogen fixation (Liu et al., 2014; Wang et al., 2018). Peck et al. (2006) studied this subject using the rhizobia *S. meliloti* and the leguminous plant alfalfa. These authors demonstrated the importance of NodD, a protein resulting from the expression of *nodD* gene, by expressing *nod* genes from other rhizobia species in *S. meliloti* without endogenous NodD activity and adding different flavonoid inducers. During the chemical synthesis of Nod factors, they are modified using, apart from other compounds, different kind of sugars, acetate or by varying the saturation degree of the acyl tail. This whole series of changes have a direct relation in the specificity legume-rhizobia (Perret et al., 2000).

After been released the Nod factors, they are perceived on root epidermis by specific receptors responsible of a cascade of molecular signals that result in the infection by bacteria and the posterior nodulation process. As an example, in model plants such as *M. truncatula* and *Lotus japonicus*, two LysM domain receptor-like kinases viz. Nod factor perception (NFP)/NFP5 and Lysin Motif Receptor-like kinase3 (LYK3)/NFR1 (Moling et al., 2014) have been described. LysM are a family of proteins that acts as receptors of Nod factors and other proteins belonging to the surface of bacteria (peptidoglycans from the bacterial cell surface) (Oldroyd, 2013). The transmembrane LysM receptor kinase called EPR3 is an example of the implication of these receptors in nodulation. Kawaharada et al. (2015) identified one gene (*Epr3*) in *L. japonicus*, involved in the step of rhizobia infection during the nodulation. They observed that the expression of the mentioned gene is inducible and dependent of Nod factors and therefore, involved in the specificity cited above. However, in the symbiotic processes, there are several other complexes of cell-surface receptor kinases depending on the plant species, the microorganism to be detected and/or the nature of that interaction (positive or negative). All of these variables have been analyzed by Zipfel and Oldroyd (2017) in a complete review in which the cascade of activation of genes during symbiosis and other root-microorganism interactions are also describe on detail.

Nitrogen fixation is not an exclusive phenomenon of the sybiotic association legume-rhizobia. The small genus of non-legume plants *Parasponia* (family Cannabaceae), in symbiotic association also with rhizobia, and actinorhizal plants (a group of more than 200 species of dicotyledonous plants belonging to eight families) with *Frankia* bacteria species are also capable of fixing nitrogen (Abdel-Lateif et al., 2012; Abdel-Lateif et al., 2013; Berry et al., 2015). As described previously, during nodulation, chalcone synthase has an essential role in *M. truncatula*. The same result was achieved by Abdel-Lateif et al. (2013) in *Casuarina glauca*, an actinorhizal plant, during nodulation with *Frankia* strain CcI3. By silencing chalcone synthase using RNA interference, it was found that the production of a specific flavonoid decreased and as consequence, the number of defective nodulation increased. The silencing effect was reverted adding naringenin.

8.1.2 Root exudates and plant growth promoting rhizobacteria

However, the rhizobacteria-root exudates relationship is more than just flavonoids, legumes, nodulation, etc. Other bacteria without the capability of developing nodules and also not with the capacity of fixing nitrogen are as well strongly influenced by root exudates. By using these exudates the plant can influence the bacterial gene expression, particularly for genes encoding on plant-beneficial aspects (Vacheron et al., 2013). These bacteria, but not those with deleterious (phytopathogens) or neutral relationship, could be grouped in a broad sense in PGPR. This is a heterogeneous group of rhizobacteria well known for providing direct (e.g. stimulation of root growth) and indirect (e.g. exerting biocontrol activity through antibiosis, competition or induction of systemic resistance) beneficial effects on plant growth (Lugtenberg and Kamilova, 2009; Backer et al., 2018). The starting point in the colonization of roots by rhizobacteria is the movement as response to gradients of specific substances released by roots through their exudates. These compounds trigger a set of genetic and biochemical responses to activate the bacterial movement towards the root surface. One of the most studied PGPR genera is *Pseudomonas* (Lugtenberg et al., 2001; Lugtenberg and Kamilova, 2009; Mercado-Blanco, 2015) in which several examples as regards this mobility are described (Capdevila et al., 2004; Barahona et al., 2016; Blanco-Romero et al., 2018), like the gram-negative bacteria *Pseudomonas fluorescens* WCS365. This rhizobacteria with high rates of colonization in tomato, shows chemo attraction to root exudates principally due to

the presence of organic acids such as malic and citric acids (de Weert et al., 2002). Through the construction of mutants in the *cheA* gene, which controls flagella-driven chemotaxis, de Wert et al. analyzed the behavior of this bacteria and the role of chemotaxis during colonization. Mutants were abnormal in flagella-driven chemotaxis but not in motility. The co-inoculation of the wildtype and the *cheA* mutant resulted a reduction of the root colonizing ability showing that chemotaxis is an important characteristic for being more effective during the colonization process. An example of gram-positive rhizobacteria can be found in *Bacillus amyloliquefaciens* FZB42 (Fan et al., 2012). The transcriptomic analysis performed as a result of the application of maize root exudates revealed significant altered expression levels (up or down regulation) in the presence of root exudates (8.2% of the whole transcriptome). From these, an important number of induced genes were related with motility and chemotaxis. Similarly, Xie et al. (2015) for a better understanding of plant-PGPR interactions, analyzed the variations in the transcriptome for the rice seedlings-*Bacillus subtilis* OKB105 pair. In this case, the 3.8% of the transcriptome showed significantly altered expression levels. Curiously, in these experiments, genes associated to chemotaxis and motility shown down-regulation, like in the experiments reported by Mwita et al. (2016) studying the effect of maize root exudates on *Bacillus atrophaeus*. By sequencing the whole genome of several bacteria (rhizobia and PGPR), relevant information about the existence of several chemotaxis systems in which a high number of chemoreceptors has been provided (Scharf et al., 2016). These chemoreceptors allow bacteria to interact with other micro/macroorganisms (Krell et al., 2011) through the detection of a high variety of signals which trigger several responses affecting their behavior and functions (e.g. motility). An example of the importance and the high number of chemoreceptors can be found in the experiments performed with the pair plant-PGPR. *A. thaliana*-*B. subtilis* strain NCBI 3610, demonstrating the attraction of this bacteria by root exudates. This activity was mediated by two characterized chemoreceptors, named as McpB and McpC, and the orphan receptor TlpC. However, mutant strains with absence of these receptors also colonized the roots, suggesting the implication of another receptors which are still not characterized that could be overlapping their activities. It was also demonstrated the requirement of a functioning chemotaxis signaling pathway for a correct root colonization in the first hours after inoculation. Oku et al. (2012, 2014) identified chemotaxis sensory proteins for amino acids in *P. fluorescens* pf0-1 and their participation in both, the chemotaxis mediated by tomato root exudates and the root colonization. Further and more detailed information related with bacterial chemoreceptors can be found in Lacal et al. (2010), with a classification of chemoreceptors as regards the size of their ligand binding regions, or in Ortega et al. (2017) in which are addressed models devoting greater attention to the type of ligand binding domain for different bacteria. All these functions (chemoreception, motility, etc.) has been observed during root colonization, the aggrupation on multicellular communities, called biofilms, for a long-lasting attachment of the bacteria to the root surface (Allard-Massicotte et al., 2016). Chen et al. (2012), studying *B. subtilis*, demonstrated the implication of tomato root exudates in the formation of biofilms. In this pair, a sensor kinase called KinD, necessary for an efficient biofilm formation, is stimulated *in vitro* by small molecules such as L-malic acid, present in root exudates. However, it is unlikely to be the only molecule that triggers this reaction. Similarly, for *A. thaliana*-*B. subtilis*, biofilms formation was triggered by certain plant polysaccharides which induce the transcription of a matrix of genes. Many of these genes are implicated in biofilm formation under *in vitro* conditions and in the colonization of *A. thaliana* roots (Beauregard et al., 2013).

As discussed above, root exudates have a clear effect among others, on microbial attraction. Once the microorganism is in the rhizosphere, there are multiple possibilities. The bacteria can use the exudates as a carbon source, developing a close interaction with the host plant such as the nodulation, biofilm formation, etc. However, one of the questions less studied has been, perhaps, how root exudates influence in the antimicrobial activity of bacteria inhabiting the rhizosphere. Jousset et al. (2011) explored this interesting aspect performing an elaborated experiment in a split-root system. In this study, plants of barley were grown with part of the radical system in a pot and the rest in other. One of them was inoculated with the pathogen *Pythium ultimum* and the other with the biological control agent *P. fluorescens* CHA0. The contact of the pathogen with part of the root triggered a systemic reaction in the plant altering the composition of root exudates in the full radical system. As a result, the beneficial *Pseudomonas* in the other pot, increased the expression of the antifungal gene *phlA*, necessary for the production of 2,4-diacetylphloroglucinol (DAPG). Meanwhile, de Werra et al. (2011). identified a considerable number of low molecular weight compounds, some of them being part of root exudates, with an effect over CHA0. Some of these compounds showed modulation of gene expression for the production of DAPG, pyoluteorin, or both metabolites, which have antibiotic activity.

8.2 Root exudates shapes the rhizobiome

As cited in introduction section, exudates composition and therefore their modulation capacity over the rhizobiome depends on several biotic and abiotic factors, with evidence of substantial differences on these microbial communities

between species (Pausch and Kuzyakov, 2018), cultivars (Szoboszlay et al., 2015) and accessions, as shown by Mönchgesang et al. (2016), studying *A. thaliana* as a plant model. Stress conditions modify the exudates composition of the plant and therefore the shaping capacity (Fuchslueger et al., 2014), but there is a lack of studies analyzing stress conditions, exudates response and microbiomes together. In short, rhizosphere microbial communities are affected by a dynamic root exudate composition along their life that is genetically programmed (Chaparro et al., 2013). This knowledge could help in the prediction of the microbial communities as revealed by Zhalnina et al. (2018) in their comparative genomics and metabolomics study.

Molecular mechanisms involved in the regulation of plant exudates are still far from being understood, but the study of mutants in model plants like *A. thaliana* or *Brachypodium distachyon* is resulting in an increasing source of the knowledge (Lebeis et al., 2015; Kawasaki et al., 2016). In this context, plant immune system plays an essential role in the modulation of microbial communities. Jasmonic acid and salicylic acid, key in the defensive responses in *A. thaliana* proved to shape the bacterial communities in mutant accessions (Carvalhais et al., 2015; Lebeis et al., 2015). As well, other evidences reveal how plant nutrition pathways, directly related with plant defense, have a great potential in the recruitment of beneficial microbes (Stringlis et al., 2018). In this study, Stringlis et al. (2018) describe the importance of the MYB72 transcription factor as a main regulator in the secretion of coumarin under iron starving conditions and help in the attraction of beneficial bacteria. This coumarin release also is produced in phosphate-starvation conditions (Ziegler et al., 2016). Future application of the new advances in molecular technics like new approaches in tRNA sequencing (Schwartz et al., 2018) or NGS will contribute for a better understanding of the cross talk plant-microbe.

8.3 Future outlook

The exponentially growing knowledge on plant-microorganisms interaction and the important role played by plant exudates in this relationship would not be possible without the development of new technologies and bioinformatic tools capable of processing the increasing amount of output data. As mentioned before, the expanding understanding about the intense and close relationship that takes place between plant and microorganisms has made that some researchers describe this assemblage as a unit, as an holobiont (Vandenkoornhuyse et al., 2015). Future understanding of the mechanisms displayed by plants and microorganisms and their cross talk will grow in parallel with the technological evolution, which will ultimately determine the speed and detail level of this scientific progress.

One of the mayor goals in understanding the mechanism regulating plant-microorganism cross talk, is to predict and manipulate the plant rhizobiome, enabling the full potential development of plant crops. Hirsch et al. (2013) already proposed the use of new available technologies to genetically modify the plants, making possible to alter the supply of rhizodeposits to microorganisms. But to do that is also important to take into account that exudates have a differential production and distribution in roots, being the most important zone the root apices. The availability of root exudates decreases, the further we are from this zone, also favoured by microbial mineralization (Dennis et al., 2010).

Defined this capacity of modifying the microbiome through rhizosphere engineering (Ahkami et al., 2017; Wallenstein, 2017), the approaches to modulate the rhizosphere microbiome are: i) via synthetic microbial communities, which is really complex since in the co-culture of strains many variables must be taking into account (Großkopf and Soyer, 2014); or ii) via re-designing the plant metabolism using CRISPR or iii) other gene editing tools (Butt et al., 2018). Last two approaches will modify the root exudates profile and thus, affecting on the microbiome. The control on the exudates will allow the recruitment of beneficial microorganisms, leading to the improvement of plant defense against biotic and abiotic stress, rising crop productivity and reducing the phytochemical inputs contributing therefore to a more sustainable agriculture.

A large amount of data and knowledge is still needed to achieve this level of understanding. Two main convergent research approaches and technological tools are being developed:

- i. The development of new sequencing technologies and the improvement and update of the already on use, allow a better definition of the microbial communities and lower sequencing costs leading to a rising amount of inputs on the databases. In this direction, meaningful advances have been made in the last years thanks to the new and cheaper sequencing technologies like pyrosequencing (Rascovan et al., 2016), allowing a higher definition of microbiomes, their distribution, diversity and evolution.
- ii. On the other side, the use of “Omics”. Describing genomics, metagenomics, metaproteomics, metatranscriptomics and metabolomics by Biswas and Sarkar (2018) as the five fingers modern molecular tools, the use of them with next-generation sequencing techniques and novel bioinformatic approaches will provide a better understanding of plant-microorganisms relationship and a new perspective as a holobiont.

The increasing genomics research, used among others to study the structure and function of genomes of the microbiomes has enabled to create a Functional Genomics Database characterize candidate genes that support bacteria in adapting to plant environments (Levy et al., 2018). The evolution of NGS (Kumari et al., 2017) and future application of the new advances in molecular technics like new approaches in tRNA sequencing (Schwartz et al., 2018) will contribute for a better comprehension of the plant-microbe interaction. On the one hand, metagenomic tools use different genetic materials for detecting microorganisms, also the unculturable ones (Cúcio et al., 2018). On the other hand proteomics and transcriptomics are focused to shed light on the structure, function and components of proteins and RNA molecules (Biswas and Sarkar, 2018).

The linkage of metabolomics and genomics on model plants is basic to improve the genetic base on exudates and bacterial uptake preferences (Mönchgesang et al., 2016; Zhalnina et al., 2018) and thus how to control them. The effect of the exudates regulation on the next plant generation (Hu et al., 2018) is of a high interest, to evaluate possible secondary effects.

The progress in the comprehension and control of the composition of root exudates will provide an important tool to modulate the rhizobiome in order to recruit beneficial microbes and enhancing plant defense mechanisms. Therefore, the use of the new technics will provide mechanisms allowing the delivery of compounds in zones depleted of root exudates and to modify both plant and microorganisms traits developing new crop management strategies (Hirsch et al., 2013; Haldar and Sengupta, 2015).

8.4 Conclusions

The current review exposes the state-of-the-art molecular studies as regards the implication and great potential of root exudates in the interaction plant-microorganisms. The actual knowledge and the future milestones of a broad research field, in which every new finding raise even more questions about the limits of the plant exudates application.

The great boost performed on research about root exudates in the last decade just open the door of what can be discovered in the next one. The development of the molecular technologies and bioinformatics, faster than expected, and the great potential of root exudates in the improvement of the crop productivity and the reduction in the use of phytochemicals by the application of rhizosphere engineering is creating a totally new horizon in agriculture that possibly will allow to overcome the actual limits of crop production.

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Chapter 9

Insight into plant-bacteria-fungi interactions to improve plant performance via remediation of heavy metals: an overview

Solmaz Kazemalilou¹, Nasser Delangiz², Behnam Asgari Lajayer¹ and Mansour Ghorbanpour³

¹Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran, ²Department of Plant Biotechnology and Breeding, Faculty of Agriculture, University of Tabriz, Tabriz, Iran, ³Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran

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9.1 Introduction

Intensive industrial activities have released so much contaminant such as heavy metals (HMs) around the world. (HMs) environmental contamination is one of the most challenging issues globally. They can persist in the environment for a long time and bring up a lot of serious threat to human and other organisms on the planet (Asgari Lajayer et al., 2017a, 2019a). HMs such as Cd, Zn, Cu, Ni, Pb, Hg and Cr are often identified in industrial wastewaters. Smelting, petroleum industries, mining, tanneries battery production, metal plating, pesticide and painting production are some of the most metal contaminating industries (Ngah and Hanafiah, 2008; Asgari Lajayer et al., 2017b, 2019b). Agricultural activities such as consuming pesticides and fertilizers also release HMs constantly resulting in contaminated soils and crops. Other anthropogenic sources can include intensive use of pesticides, herbicides, fungicides, and fertilizers. HMs are also present natively in the soil from volcanic eruptions, the weathering and pedogenetic mechanisms of parent materials and hydrothermal release of metals at levels that are considered as trace elements ($<1000 \text{ mg kg}^{-1}$). HMs that comes from anthropogenic sources are generally more bioavailable than pedogenic, or lithogenic ones (Navarro et al., 2008). These sources add various organic and inorganic contaminants to the soils, and pose serious threats to food security issues and other potential risks to human health because of soil-to-plant transfer of contaminants (Shen et al., 2002; Khan, 2005).

Furthermore, metals cannot be broken down or converted into other substances, and when their concentrations inside the plant cells increase above optimal threshold, it may cause direct damage to cellular structures and toxicity (because of oxidative stress induced by ROS) and inhibit the activity of cytoplasmic enzymes. HMs can destroy the balance between antioxidants and oxidative stresses. Also, HMs can replace essential elements and this can result in poisonous effect (Taiz and Zeiger, 2002).

9.2 Remediation technique and its applications

Remediation is an innovative environmental friendly approach to remediate soil (Mitch, 2002), which can be used to remediate both organic and inorganic contaminants (Salt et al., 1998). The contaminated soils including areas of agricultural fields and their remediation is a serious issue (Khan, 2005). As trace elements cannot be degraded they are considered as non-biodegradable. They may be accumulated in the tissues of living organisms, therefore they only moved from one place to the other (Kumpiene et al., 2008). Conventional methods of bioremediation are based on the physical and chemical approaches. Combination of them can be used for better efficiency. However, these methods can be more expensive, time consuming and damaging for the environment, compared with the biological approach (Ahmadpour et al., 2012).

9.3 Phytoremediation: definition, advantages and limitations

Phytoremediation is one of the remediation techniques and defined as the process, which uses green plants and their associated microorganisms to assimilate, transform, metabolize, or detoxify metals, hydrocarbons, pesticides, and chlorinated solvents (various toxic inorganic and organic compounds) from the contaminated environment (soil, water, groundwater, and air) to render their health hazards. It is considered as a more economical and promising technology for the remediation of polluted soils (Raskin et al., 1997). Phytoremediation is a general term and includes several processes. The relationship between Plants, soil and microbes is vital for phytoremediation processes (Mahmoud and Hamza, 2017). Some organic compounds can be degraded and bioremediated in the soil but as the number of related microbes are little this is a slow process (Brookes and McGrath, 1984). The number of microbes in rhizosphere is much more (10–1000 times) than bulk soil, so plants are important in remediation (Gaskin et al., 2008). Plants uptake some metals such as Cu, Fe, Mo, Zn and other, because there are essential for plants health. They act as a part of many enzymes and proteins in optimum amount. Plants need to accumulate some of them inside themselves. Some of metals, such as As, Pb, Cr, and Hg, are not essential for plants' growth and health (Mitch, 2002; Gaur and Adholeya, 2004).

The toxic concentration of the essential metals or the non-essential toxic ions in the soil is strongly poisonous to metal sensitive enzymes, resulting in growth inhibition and death of the organism and can lead to toxicity symptoms in plants. Some plant species such as willow (*Salix viminalis* L.), maize (*Zea mays* L.), Indian mustard (*Brassica juncea* L.), and sunflower (*Helianthus annuus* L.) have a high tolerance to HMs and ability to grow in metalliferous soils. Such plants can be used to clean up heavy metal-contaminated sites (Mahmoud and Hamza, 2017). The term phytoremediation is derived from the Greek phyto which means “plant”, and the Latin suffix remedium which means “able to cure” or “restore”. It can be used for a wide range of organic and inorganic contaminants. The positive aspects of phytoremediation are as follow: (1) cost-effective, compared with other remediation techniques; (2) environment-friendly and low disturbance to the environment; (3) It can be used for most of HMs, and (4) ease of use (Mahmoud and Hamza, 2017). Also, the use of plants for bioremediation is accepted by public (Salt et al., 1995). Because the presence of plants make beautiful views and inhibit the soil erosion and soil dust blowing (Raskin and Ensley, 2000). Plants species that are used for phytoremediation should: (1) have high capacity uptake of pollutants; (2) have high capacity to grow fast in contaminated condition and (3) to be controlled easily. It should be considered that plants are hugely different in phytoremediation ability (Singh et al., 2003). Of course phytoremediation has its limitations. This method also has some limitations. For instance, the number of HM tolerated species are so little. Moreover, most of these plants tolerate individual HMs. The identified metal hyperaccumulator plants Such as *Thlaspi caerulescens* and *Alyssum bertoloni* produce relatively low amounts of biomass due to small size and slow growth and most of them can be used in their natural habitats alone (Kamnev and van der Lelie, 2000). Phytoremediation as an environmental cleanup technology is a relatively slow process. It may take several years or longer to reduce metal contents in soil to a safe and acceptable level and it is only applicable to surface soils (Chaudhry et al., 2005). Phytoremediation has some disadvantages such as geological and climatic limitations, potential toxicity of the pollutants to plants and potential for the contaminant to enter the human food chain (Truu et al., 2015). Therefore, the use of plants in phytoremediation is restricted due to these reasons.

On the other side, some plants can accumulate huge amount of HMs inside their specific cells. These plants are known as hyperaccumulators. For the phytoremediation of HMs from the soil, we should identify fast growing plants which produce extensive root systems. Some of plants such as *Chrysopogon zizanioides* and *Cannabis sativa* are fast growing and HMs tolerant but cannot be known as hyperaccumulators, however, they can grow in HM contaminated soils. This species can easily be grown in different climates and their biomasses can be used for non-food industries. So, they can be considered as ideal plants for HMs bioremediation (phytoremediation) (Khan, 2005). Almost 400 species were identified as hyperaccumulator, which can accumulate HMs such as cadmium, nickel, manganese, arsenic, zinc (Vara Prasad and de Oliveira Freitas, 2003; Subhashini and Swamy, 2013). However, most of them are slow growing and have small size so are not appropriate for phytoextraction.

9.3.1 Chelator and acidifying agents enhanced phytoremediation technology

In order to enhance the speed and quantity of metal uptake by plant, soil amendments with metal chelating agents such as synthetics (ammonium thiocyanate) and natural zeolites have shown promises. EDTA, HEDTA, DTPA, EGTA, NTA, citrate, oxalate, malate, succinate, tartrate, phthalate, salicylate, acetate and hydroxylamine etc. have been used to artificially make metals bioavailable and absorbed by plant roots. The type of chelate and their application are important factors. It has also been suggested that if the plant biomass can be increased by metal chelating agents, then metal phytoextraction can be increased more than the plant can take up normally. Chelates are used to increase the phytoextraction of a number of metal pollutants including Cd, Cu, Ni, Pb, and Zn (Vara Prasad and de Oliveira Freitas, 2003).

Some plants can accumulate HMs by chelating agnates. This is known as “chelate-induced hyperaccumulation”. When chelate-induced hyperaccumulation mechanism is applied, metals on the contaminated site are initially immobilized to allow for quick establishment and growth of an agronomic crop such as corn. After producing enough amount of biomass, chelating materials are added to the soil to promote the scavenging of large amounts of metal. Then HMs can be absorbed by the plants root, and then they can be translocated to above ground shoots where they can be accumulated in high concentrations. After death, plants are harvested and removed from the site. This technique is opposite to the phytoextraction. As in phytoextraction plants are exposed to non-toxic levels of HMs gradually and as the plants grow they can accumulate HMs. The massive release of chelating agents can leads to HMs beyond the reach of plants as they can be washed out of the shallow layers of the soil to the deeper parts. In this case, HMs cannot be recovered through phytoremediation (Vara Prasad and de Oliveira Freitas, 2003). However, to make HMs more available in the soil, experts can use different fertilizer salts and acidifying agents. These chemicals can liberate HMs from the solid phase of the soil or increase the solubility of precipitated HMs (Lasat et al., 1998). The improvement of phytoextraction through using the acidifying agents to the soil is well proved. Metabolites such as organic and inorganic acids, elemental sulfur and NH_4 -containing fertilizers are some of these acidifying (Vara Prasad and de Oliveira Freitas, 2003). Moreover, the plant growth regulators (PGRs) increase growth rate and biomass production, therefore, their application can increase the efficiency of phytoremediation (Khan, 2005). For instance, the Fe bioavailability can be increased through IAA. It is involved in the secretion of siderophores (Patten and Glick, 1996). Plant growth promoting rhizobacteria (PGPR) also can produce IAA in plants. This results in promoted uptake of Ca, Fe, K, Zn, and P in crop plants. *Acinetobacter* and *Pseudomonas* are two of these bacteria. Fertilizers should be consumed under deficiency conditions to help plants to produce higher biomass and accumulate more HMs. Usefulness of PGPR is however, restricted under nutrient deficient conditions. Fertilizers help plants to produce higher biomass and to accumulate more metals. For finding more efficient hyperaccumulator plants, further studies should be conducted to find out the best combination of PGPR, plant and soil type (Khan, 2005).

9.4 Phytoremediation technologies in removing soil metals

Depending on the process by which plants are removing or reducing the toxic effect of contaminants from the soil, phytoremediation technology can be categorized as follows: (Chaudhry, 1998; Mahmoud and Hamza, 2017).

9.4.1 Phytodegradation or rhizodegradation

Some of the organic contaminants (non-volatile compounds) can be directly degraded and completely mineralized by plant enzymes and their associated microbes (Alkorta and Garbisu, 2001; Wild et al., 2005; Mahmoud and Hamza, 2017). Many plants produce, and often release exudates (such as sugars, amino acids and organic acids as secondary metabolites and an energy source for microbial growth) or enzymes into the root zone, that can degrade a wide range of complex

organic compounds by stimulation in the microbial and fungal degradation of organic pollutants. However, inorganic pollutants are non-biodegradable (Kumpiene et al., 2008). The major reason for the increased biodegradation of pollutants in the rhizosphere region with combination application of plants and microbes is maybe the increase in the population and activity of microorganisms. The release of root exudates by plant provides a nutrient-rich environment in which beneficial microbial growth and activity is stimulated. It also can improve the physical /chemical properties of polluted soil, and increase contact between the root-associated microbes with and the pollutants in the soil (Kuiper et al., 2004).

9.4.2 Phytostabilization

Phytostabilization is reducing the bioavailability and the mobility of the contaminants in the soil or water by plants (Kumpiene et al., 2008; Mahmoud and Hamza, 2017). The purpose of this method is not removing contaminants from the nature, but is restricting their movement in the environment. The polluted soil should be ploughed before the cultivation of proper plants, then lime, fertilizers or other amendments are added to the soil. This way, HMs can be inactivated for long time but not permanently. Manganese or iron oxyhydroxides, phosphate fertilizers, organic matters, and artificial clay minerals, or mixture of them are some of the best amendments to inactivate contaminants in the soil. The plants chosen for phytostabilization should be weak in translocating HMs from the medium to the tissues, so, the plant after complete growth can be used by human. Consuming the phytostabilizing plants by human, eliminates the necessity of their removal from the nature as hazardous plants (Flathman and Lanza, 1998). Although the phytostabilization is most efficient in soils which have fine-texture with high amount of organic matters, it can be used for any high contaminated soils.

9.4.3 Phytoextraction or phytoaccumulation

Extracted, transported and accumulated of metals into harvestable plant parts and sequestration in plants. This method is applicable for many regions with low to moderate levels of HMs in shallow depth of the soil (Luo et al., 2005). This approach is ten times cheaper than the conventional methods to remediate pollutants. The plant roots absorb metals from soil and translocate them to the shoots. In the case of inadequate amount of HMs to be absorbed by plant root, mineral nutrients or acidifying agents may be added to liberate HMs and make them available for plants root uptake (Zia et al., 2010). After complete plant growth, above ground organs should be collected from the site because of permanent removal of HMs.

Many of hyperaccumulators such as *Alyssum bertolonii* and *Thlaspi caerulescens* which are known as efficient hyperaccumulators, grow slowly and produce low biomass. Therefore, for effective bioremediation, these plants should be cultivated in the contaminated site (Khan et al., 2000). In general, the ideal plants for phytoremediation must be fast growing, competitive, and tolerant to HM (Vara Prasad and de Oliveira Freitas, 2003). Trees are interesting candidates for phytoremediation, as they have extensive root system; however, their ability in accumulating HMs is limited (Glick, 2010). The contamination extent, the bioavailability of HMs, the uptake, transport and accumulation, root system development, and HMs tolerance of the plant are key factors that indicate the efficiency of the phytoremediation (Ernst, 1996; Pilon-Smits, 2005). Tolerating environmental conditions such as soil features (salinity, pH, humidity, etc.), diseases challenge, the ability of the establishment may be some of the other characteristics of the plants which can affect their remediation efficiency (Murtaza et al., 2014).

9.4.4 Phytovolatilization

In this process, low molecular weight compounds are imported by the roots system then are transported to the leaves and then eventually released into the air through the evapotranspiration process. Phytovolatilization can be used for both organics compounds, such as volatile organic carbons (VOCs) and inorganic compounds which are present in volatile form such as Hg, Se, and As (Pilon-Smits, 2005; Lim et al., 2016). Through this method, toxic compounds can be transformed to less harmful ones. For instance, Se in volatile form, such as dimethylselenide is less toxic (about 1/600 to 1500 times) than the inorganic form (de Souza et al., 2000). As the gaseous form of elements cannot be deposited again at least near the contaminated site, Se and Hg volatilization is a permanent way to remove them from the soil (Pilon-Smits, 2005). Moreover, this method does not need complex management. In this method, the biomass deposition is not needed. Moreover, planting can prevent the erosion of the soil (Heaton et al., 1998). However, phytovolatilization has itself draw backs. For instance, when gaseous from of elements are released to the environment, there is no control over their migration. Mercury can precipitate into the lakes, oceans and transformed to the methyl-mercury form (Mahmoud and Hamza, 2017).

9.4.5 Rhizo-filtration

In situ or ex situ absorption, concentration and precipitation of HMs through the root system in both contaminated waters and soils is rhizo-filtration. However, terrestrial plants are preferred for this system as they produce root biomass more extensive than aquatic plants. Moreover, they often have fibrous root systems for metal sorption. The metal such as Cu, Cr, Cd, Zn, Ni, and Pb are some of HMs which can be removed through rhizo-filtration approach from the environment. Some plants were studied for their rhizo-filtration of lead and reported that sunflower is the best one (Raskin and Ensley, 2000). In this system, HMs are not translocated to the shoots. It is known as the most important positive feature of rhizo-filtration.

9.5 Heavy metal phytoavailability

Plants can absorb phytoavailable contaminants via the roots. The phytoavailability is a kind of complex phenomena which depends on the many factors (Peijnenburg and Jager, 2003). Soil properties (such as pH and texture) and the redox potential of the root zone are some of the important features which can affect the potential of phytoavailability. The concentration of bioavailable contaminants can be affected by some biological, chemical and physical processes (Pilon-Smits, 2005). The HMs solubility can be enhanced by increasing the soil acidity or organic ligands. Moreover, chelating agents such as ethylenediaminetetraacetic acid (EDTA), pyridine-2-6-dicarboxylic acid (PDA), hydrochloric acid, nitrilotriacetic acid (NTA), nitric acid can be used to increase the solubility of the HMs (Romkens et al., 2002). However, the excess amount of them increases the leaching of HMs into the ground waters and their phytotoxicity. To decrease their phytotoxicity, lime or organic matters can be added to the soil (Pilon-Smits, 2005).

9.6 Assisted phytoremediation by microorganisms: rhizoremediation

Rhizoremediation, the use of plants and associated microorganisms is a promising technique to enhance the efficiency of contaminants extraction. This beneficial association can raise the availability of the compounds by the microorganisms and facilitate the plants in the extraction and removal of such compounds (Chaudhry et al., 2005). It is beneficial to both, once the plants supply nutrients for microorganisms which in turn grow and develop enhanced capacity of degradation by plants thus reducing the phytotoxicity of the contaminated soil (Siciliano and Germida, 1998); however, little scientific data are available about this synergism between plants and microorganisms (Kavamura and Esposito, 2008).

9.6.1 Plant growth-promoting bacteria and rhizoremediation

Bioremediation as emerging approach uses various organisms to degrade, detoxify, and remediate pollutants. Microorganisms in the root zone (rhizobacteria) can act synergistically with the plants. They can facilitate nutrient absorption by plant and stimulate plant growth (Yang et al., 2009). The toxic elements can be negatively affect some soil processes. For instance, the hazardous level of HMs can decrease the number of microbes and the rate of nitrogen fixation. However, the extend of the effect of HMs depends on different factors such as temperature, pH, amount of organic inorganic content of the soil (Giller et al., 1998). Using organic wastes can alter the soil's microbial activity. The improved microbial activity results in improved HMs uptake through the plants (Chen et al., 2015).

The most endophytic bacteria are recently used to improve phytoremediation strategies. Endophytic bacteria are known to have pathogen control and plant growth promoting abilities and they exist in most plant species. Furthermore, they often belong to genera mostly found in the agriculture soils, including *Burkholderia*, *Azospirillum*, *Pseudomonas*, and *Bacillus* (Ryan et al., 2008). The efficiency of bioremediation depends on the nature of the organisms used, the dominant environmental factors at the pollutant soil, as well as the level of the contaminants in that soil (Azubuike et al., 2016). The use of soil bacteria (often plant growth-promoting bacteria) as adjuncts in metal phytoremediation can directly promote the growth of plants in the presence of high metal concentrations (Glick, 2010). Plant growth promoting bacteria (PGPB) may fix atmospheric N₂ gas into organic N forms and supply it to plants; synthesize siderophores that serve as Fe-chelating agents; produce plant growth regulators such as auxins, cytokinins and gibberelins and improvement in plant nutritive status such as phosphorus and other mineral nutrients, or siderophore-producers (Amora-Lazcano et al., 2010), alter the valence electrons of heavy metals and then their chemical precipitation. Some rhizobacteria can produce Indole-3-acetic acid (IAA) which increases root growth and surface area, increasing

availability of soil nutrients and thereby enhancing root exudates which provide the more nutrients for activity of rhizospheric bacteria against stress conditions (Ojuederie and Babalola, 2017).

Bacterial siderophores can indirectly alleviate trace element toxicity by increasing the plant iron supply and eliminating iron deficiency (Burd et al., 1998, 2000; Weyens et al., 2009). Phosphate-solubilizing microorganisms can convert phosphorous to available forms for plants under stress conditions. Most of PGPB synthesis the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which reduce poison ethylene levels in plants under heavy metal stress conditions (Ojuederie and Babalola, 2017). Sulfate-reducing bacteria such as *Desulfovibrio desulfuricans* reduce SO_4^{2-} to hydrogen sulfide which then reacts with heavy metals to precipitate highly insoluble forms of metal sulfides (Chibuike and Obiora, 2014). The presence of such bacteria in plants increases phytoremediation efficiency, and decreases demand for further fertilization (Afzal et al., 2014). Rhizosphere bacteria are considered to be useful in phytoremediation, since they promote plant growth by production of siderophore and phytohormones, mobilize mineral phosphate, immobilization of heavy metals through bioaccumulation, biotransformation to less soluble forms, chemisorption and biomineralization of heavy metals (Iqbal and Ahemad, 2015). Therefore, the interaction of metal-microbe affects degradation of pollutants and bioremediation. Use of beneficial microorganisms such as endophytes or rhizosphere bacteria increases plant growth and biomass production of hyperaccumulating plants (Cabello-Conejo et al., 2011). Higher biomass production is correlated with increased remediation. Whiting et al. (2001) compared the effect of rhizosphere bacteria on Zn accumulation by a hyperaccumulator (*Thlaspi caerulescens*) and a non-accumulator (*Thlaspi arvense*). Their results showed that the rhizosphere microflora enhanced biomass production and Zn uptake of the hyperaccumulator, whereas *T. arvense* was not affected.

Bacteria as important biosorbents used for heavy metals removal in bioremediation processes, they can tolerate severe environmental conditions and are able to grow under these conditions (Srivastava et al., 2015). They may indirectly stimulate plant growth and development by antibiotic production and control of phytopathogens, induced systemic resistance, production of fungal cell-wall lytic enzymes, and competition for binding sites and nutrients on the root surface (Amora-Lazcano et al., 2010). A group of bacteria is better than single one for efficient bioremediation. For instance, Kang et al. (2016), observed that the bacterial mixtures (*Enterobacter cloacae* KJ-46 and *E. cloacae* KJ-47, *Sporosarcina soli* B-22, *Viridibacillus arenosi* B-21) is more efficient in the bioremediation of group of HMs (Pb, Cu, and Cd) in compare with single bacterium culture.

The soil microbes have an important role in the nutrient recycling, improvement of soil structure, degradation and detoxification of harmful chemicals, management of plant diseases and promote plant growth (Iqbal and Ahemad, 2015). Thus, bacteria can increase the capacity of plants to remediate or reduce the phytotoxicity of the polluted environment.

In addition, plants can secrete root exudates to rhizosphere, then by increasing of ion solubility may improve the remediation activity of soil microorganisms associated with plant roots. Rhizobacteria have known to have some characteristics that can make changes in bioavailability of heavy metals through the release of chelating agents, acidification of the rhizosphere and changes in the redox potential (Iqbal and Ahemad, 2015).

9.6.2 Mycorrhiza-assisted remediation (MAR)

MAR is an aspect of bioremediation that uses mycorrhiza for the treatment of contaminated soils establishing symbiotic relationships with 80–90% land plants (Khan, 2005). Besides symbiotic bacteria, symbiosis with mycorrhizal fungi has been proposed as one of the HMs tolerance mechanisms in plant through phytoremediation technology (Upadhyaya et al., 2010). The majority of mycorrhizal interactions are mutually beneficial relationship for both partners and are characterized by a bidirectional exchange of resources between plants and fungi. The mycorrhizal fungus extends the surface area of the plant's roots due to their small diameter and thus increases their ability to absorb more nutrients such as phosphate, nitrogen, potassium, sulfur, trace elements (copper, zinc and other nutrients) and water to their host plants. They can help the plant to overcome a variety of biotic (root pathogens) or abiotic stresses (drought, salinity, HMs). They can further modify contaminant bioavailability in several ways, including competition with roots and other microorganisms for water and contaminant uptake, protection of roots from direct interaction with the contaminant through formation of the ectomycorrhizal sheath, and impeded contaminant transport via increased soil hydrophobicity (Meharg and Cairney, 2000). They also have an ability to tolerate and detoxify metal ions via active accumulation, intra- and extracellular precipitation, valence alteration, and absorption of heavy metals into their mycelium and spores (Ojuederie and Babalola, 2017). In return, the host plants transfer up to 20–25% of its photosynthetically fixed carbon to the mycorrhizal fungus (Chibuike, 2013; Bücking et al., 2018).

Arbuscular mycorrhizae (AM) and ectomycorrhizae (ECM) are two types of mycorrhizae used for the bioremediation of contaminated soils. However, the use of AM as bioremediator is prevalent as they are able to colonize most of agricultural plants. Also, they can act as bioprotectors (against stresses and pathogens) and biofertilizers (Finlay, 2008). However, there are some other mycorrhizae, such as Ectomycorrhizal fungi (ECM), which can be a part of sustainable restoration or phytoremediation operations. Because ECM have symbiotic relationship with various shrub and tree species and play important role in their healthy growth (Bücking et al., 2012).

AM can produce a type of protein (insoluble glycoprotein) commonly known as glomalin, which bind HMs beyond the plant rhizosphere. Glomalin is capable of extracting Cd (0.08 mg), Cu (4.3 mg), Pb (1.12 mg) from HM contaminated soils (Gonzalez-Chavez et al., 2004). In addition, the high surface area of the AMF hyphae in the soil are an important sink for HMs. For instance, *Glomus mosseae* adsorb Cd (up to 0.5 mg per day) to its hyphae passively. The other studies proved that the uptake and immobilization rate of Pb is greater in mycorrhizal plants root in compare with non-mycorrhizal plants. It seems that fungal vesicles are involved in accumulating toxic compounds, so they may have role in detoxification process. In maize, HMs are selectively retained in the inner parenchyma cells coinciding with fungal structures. AM fungi can colonize roots of dominant plant species even in highly contaminated soils (Upadhyaya et al., 2010).

The translocation rate of contaminants from roots to the shoots of plants can be decreased by MAR. AM fungi are able to restrict the transfer of HMs from the roots to the shoots through filtration mechanism. Therefore MAR is ideal choice for phytoremediation, as plants which are usually removed and burned after phytoextraction operation can restrict the erosion rate in the contaminated region. Moreover, fungal spores can survive for long time (up to 6 years), so they can support any other crops which are planted in the region. Therefore, MAR can guarantee the fast vegetation restoration of remediated soils (Chibuike, 2013). If the plant can take minerals efficiently, it would produce more biomass, which is crucial for efficient remediation.

The MAR ability to remove the HMs effectively directly depends on the plant species which is colonized by specific fungus. It was reported that MAR can be more effective when *Trifolium repens* was cultivated in a soil which two native plants, such as *Pteris vittata* and *Coreopsis drummondii*, were cultivated (Chen et al., 2007). This combination was significantly effective in Cu poisoned soil. MAR also depends on the origin of fungi, HMs concentration and type. Therefore, the appropriate fungi should be used in the case of MAR using. Combination of MAR with adding organic compounds and phosphate rocks can enhance the rate of HM polluted soils (Leung et al., 2010). It was also demonstrated frequently that legumes have high phytoremediation potential as they produce widespread roots. *Rhizobium* improves the growth of mycelia in mycorrhizal fungi, while the fungi supplies phosphorus that helps nitrogen fixation. Therefore, the symbiotic association between these organisms indirectly increases the efficiency of remediation (Chibuike, 2013).

The ability of fungi to remediate HMs is affected by nutrients deficiencies (especially P and N) as their activity is prevented in this condition. Thus, using some amendments can improve the remediation efficiency. However, excess amount of P can prevent the growth of some fungi. So, amendments should be used cautiously. Organic amendments are recommended more than inorganic ones as they release nutrients slower (Chibuike, 2013). The alleviation effect of AM fungi has two main causes: AM can mediate the nutritional effect, and AM affects the distribution of HMs between soil, plant and fungus. Both moderate and extra level of fungi can enhance the barrier for translocating HMs from the root (Kaldorf et al., 1999; Joner et al., 2000) (Chen et al., 2007; Upadhyaya et al., 2010). AM fungi in HM contaminated soils, cope with the HM-toxicity better than AM fungi which are derived from non-contaminated soils (Weissenhorn et al., 1993).

Some studies inspect the ability of saprobes (a group of fungi with organic compounds breaking down ability) in biodegrading soil pollutants. *Trichoderma* sp. and *Fusarium* sp. are have been used in conjunction with MAR to remediate the soil polluted with HMs (Arriagada et al., 2004). Using them with mycorrhiza, makes remediation process more efficient. The amount of the HMs that is removed from the soil is dependent on the origin of the mycorrhizal fungi. For instance, Orłowska et al. (2012) reported that fungi strain which are isolated from contaminated soil are more efficient in bioremediation in compare with the fungi isolated from other regions due to prior adaptability of fungi. However, the most plants from the Brassicaceae family have not ability to establish mycorrhizal symbiosis (Marschner, 1995). Researchers have found that their exudates may have negative effects on the mycorrhizal fungi (Cardoso and Kuyper, 2006). The AM inhabits the HM-contaminated soils is more tolerant than others. Also, it can colonize the plant roots more efficiently in such soils. Thus, screening the native contaminated soil to isolate AM which are effective in remediating HMs is important. It is also suggested that the phytoremediation of heavy metal contaminated soil can be enhanced by inoculating hyper-accumulator plants with mycorrhizal fungi. The Knowledge about the interaction of AM–plant is increasing under HM-stress. However we know about the fungi-plants interaction under

HM-stress in a limited extend. little data is available the about whether there is a synergism between plant and fungal HM-tolerance. It seems that AM fungi can be more effective in bioremediating contaminant the case of plant cooperation and colonization. Because compatible organisms protect themselves to grow and reproduce (Upadhyaya et al., 2010).

9.7 Conclusion and future prospects

As a result of intensive industrialization, heavy metals accumulation results contamination of the soil. These substances led to the destruction of soil and have adverse effects to human health. Bioremediation as a promising tool to further improvement and development of the remediation of polluted soils do not have negative effects on the environment, with positive properties such as improving the soil structure, increasing uptake of nutrients and microbial activity in the rhizosphere causing to increase in plant stress resistance. Beneficial plant-microbe interactions with AM fungi and PGPBs is a suitable method for the clean-up of polluted soils and may alleviate metal phytotoxic effects and improve plant growth and nutrient uptake. The efficiency of this method of remediation depends on the rhizobial strains, species and origin of the AM fungi used, the type of plant colonized, and the type and concentration of the metals. The combination of rhizosphere microbes especially MAR and PGPBs with other methods of phytoremediation improved its efficiency and could reduce some of the problems faced during the application of both single methods. However, more researches of the selection of suitable compatible rhizobial and AM fungus strains or communities are needed in order to sustain and increase the plant root system which is compatible for rhizoremediation.

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Chapter 10

Signaling pathway of induced systemic resistance

Deepak G. Panpatte, Yogeshvari K. Jhala and Rajababu V. Vyas

Department of Agricultural Microbiology, B. A. College of Agriculture, Anand Agricultural University, Anand, India

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10.1 Introduction

Plants serve a central role in existence of life on earth, they utilize solar energy and drive all the life functions on earth. Plant roots harbor a large number of microorganisms termed as rhizobacteria which survive by using root exudates and plant lysates as nutrients. Plant's rhizosphere contains certain genera of bacteria having capacity to encourage plant growth and they are termed as plant growth promoting rhizobacteria (PGPR). PGPR can also stimulate plant defense system to cope up with various biotic stresses. Induced defensive state of plants wherein plant's defense system is preconditioned that helps to reduce disease severity. Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are two types of induced resistance mechanisms adopted by plants. SAR pathway gets activated due to prior infection by pathogens where ISR pathway is triggered by non-pathogenic rhizobacteria (Choudhary et al., 2007). Characteristic feature of induced resistance includes stimulation of dormant protection mechanisms which can be triggered during consequent attack by pathogen or insect herbivore. Such induced resistance is not only expressed at the site of induction but also visible in the systemic tissues which are spatially separated from inducer, hence the term ISR is used. Commonly, induced resistance provides improved strength of defence against a broad spectrum of pathogens (Fu and Dong, 2013). Induced resistance is controlled by plant hormone driven signaling pathways that are interlinked with each other. Unique feature of induced systemic resistance includes increased level of plant's basal resistance to various pathogens concurrently that provides benefits in natural circumstances comprising of several pathogens. Phenotypically ISR can be explained as the mechanisms in which colonization of plant by beneficial microbes induce defensive state of plant and prepare them against further infection by pathogens. Induced resistance has broad spectrum efficiency on the basis of quicker and stronger initiation of basal defence system of plants enabling them to react more efficiently under stress conditions. This enhanced immunity of plant is similar to the immunization of animals and humans. Such induction of systemic resistance by expressing basal defence mechanism is known as priming (Conrath et al., 2002).

Plant's induced resistance state is regulated by plant hormones like jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Upon pathogen infection the plants will respond by producing mixture of such alarming signals. Type of the defence response against pathogen will be determined by quantity, composition, timing and gene expression induced by signals. There exists cross talk between SA, JA and ET mediated defence response which provides a dominant monitoring prospective allowing plant to respond to attackers. In majority of the cases of induced resistance, primary attack by

pathogens or herbivorous insects are generally linked to increase in manufacturing of these hormones followed by activation of distinctive groups of defence linked genes. In many cases, it has been noticed that external application of such plant hormones results enhanced resistance (Van Wees et al., 1999). Based on host-pathogen interaction, JA, SA and ET seem to be differentially included in basal resistance.

Initial research regarding decoding the mechanisms of PGPR mediated ISR pathway focused on role of PR proteins as increased concentration of PR proteins was believed to be correlated with enhanced disease resistant state of plants. First indication about presence of PGPR mediated plant immunization was given in 1991 by three separate research groups. Van Peer et al. (1991) revealed that carnation root colonization by PGPR strain *P. fluorescens* WCS417r, displayed higher level of resistance against *F. oxysporum* in aboveground parts. Moreover, *P. fluorescens* WCS417r colonized plants displayed significantly higher production of antimicrobial phytoalexins at the site of infection by plant pathogen providing evidence that signals generated by *P. fluorescens* WCS417r in roots alert systemic plant parts for improved pathogen defence. Same way, Wei et al. (1991) in cucumber reported that plant root colonization by plant growth promoting rhizobacterial strains of *Pseudomonas* and *Serratia* showed significant reduction in anthracnose disease incidence in leaves caused by pathogen *Colletotrichum orbiculare*. In both studies, PGPR and pathogen were confirmed to have stayed spatially parted in experiments, which permit authors to accomplish improved level of resistance against disease through plant facilitated immune response called rhizobacteria-induced systemic resistance (ISR).

10.2 Difference between ISR and SAR

ISR shows broad spectrum of efficiency and therefore initially it was believed to be mechanically similar to SAR. This belief was broken apart by Hoffland et al. (1995) where he showed that ISR induced by *P. fluorescens* WCS417r against *F. oxysporum* in radish does not showed accumulation of PR gene products which is essential phenomenon of SAR. Same way, ISR induced by *P. fluorescens* WCS417r in *Arabidopsis* was also not linked with PR gene activation in systemic leaf tissue. Moreover, signaling pathways for both the resistance mechanisms are different. Experiments with transgenic *Arabidopsis* NahG plants ascertained that *P. fluorescens* WCS417r induced ISR was not seen to be correlated with accumulation salicylic acid (SA). Results provides genetic indication that ISR is facilitated by an SA-independent signaling pathway and does not correspond to higher SA levels (Pieterse et al., 1996, 2000). Similar results were evident in experiments with PGPR *P. putida* WCS358r mediated ISR. All the above evidences suggest that PGPR induced ISR and pathogen triggered SAR are being controlled by diverse signaling pathways which was supported by the divergence in efficiency range of both the pathways against extensive range of pathogens. Van Loon and Bakker reviewed rhizobacteria assisted ISR and concluded that beneficial microbes commonly induce SA independent ISR pathway in broad range of plant species. So, it is clear that SAR induced by pathogens and found to be SA dependent whereas ISR pathway is SA independent and induced by PGPR. SA mediated defence response is generally effective against biotrophic pathogens as it is associated with programmed cell death known as hypersensitive response. SA mediated SAR results in death of the pathogen infected cells (Fatima and Senthilkumar, 2015). Biotrophic pathogens are most effectively controlled by SAR pathway. Whereas SAR pathway is not effective against necrotrophic pathogens as they do not require live host instead they flourish in presence of dead tissues. In other words, SA dependent SAR pathway can accelerate the growth of necrotrophic pathogens. So JA mediated ISR pathway can effectively control necrotrophs.

10.3 Signal transduction pathway underlying PGPR mediated ISR

Signaling in ISR appears considerably more complex. It was believed that ISR pathway is having central role of PR proteins as the case with SAR defence response. This belief was broken down by the Hoffland et al. (1995) proved that *P. fluorescens* WCS417r mediated ISR against *F. oxysporum* in radish does not results from accumulation of PR gene products. Likewise *P. fluorescens* WCS417r also helped to develop ISR response against *F. oxysporum* f. sp. *raphani* and *P. syringae* ISR in *Arabidopsis* plants without activation of PR genes in systemic leaf tissue (Pieterse et al., 1996). Testing of transgenic *Arabidopsis* NahG plants that are unable to accumulate SA provided genetic evidence that *P. fluorescens* WCS417r-ISR is mediated by an SA-independent signaling pathway and does not coincide with enhanced SA levels (Pieterse et al., 1996). The same appeared to be true for the ISR-inducing PGPR *P. putida* WCS358r. It was thus concluded that rhizobacteria-mediated ISR and SA-dependent SAR are regulated by different signaling pathways. This was supported by observations that although both rhizobacteria-mediated ISR and pathogen-induced SAR are effective against a broad spectrum of attackers, their ranges of effectiveness are partly divergent.

10.3.1 Signaling of ISR pathway

It was ascertained that ISR pathway is not dependent on salicylic acid and also does not display accumulation of PR proteins in local and systemic tissues, so alternate pathway for signal transduction during ISR response was discovered to understand molecular mechanism of PGPR mediated plant defence response. To decode signal transduction pathway of ISR assisted by PGPR, an *Arabidopsis* based model system containing PGPR strain of *P. fluorescens* WCS417r is used as inducing agent and *P. syringae* pv. *tomato* DC3000 as pathogen (Pieterse et al., 2000). It was ascertained that PGPR mediated ISR needs sensitivity to jasmonic acid and ethylene and do not rely on accumulation of both the hormones and the initial signaling component of the ISR pathway is NPR1. In the *jar1* mutant having lowered sensitivity to jasmonic acid (JA), WCS417r was not capable to induce ISR proving that role of jasmonic acid in rhizobacteria mediated defence response pathway. Same way, ethylene-insensitive *Arabidopsis* mutants *etr1* and *ein2* were not expressing ISR upon priming by WCS417. ETR1 codes for an ethylene receptor and dominant *etr1* mutation resulted in lower sensitivity to hormone. EIN2 is a membrane protein which is similar to Nramp family of metal-ion transporters. It has a key part in ethylene response, unfortunately their biochemical role is unidentified. Newer ethylene insensitive mutants like *ein3–ein7* and *axr1-12* were also impaired in ISR pathway showing that ISR response needs complete signal transduction pathway of ethylene (Pieterse et al., 2000). Just like PGPR stimulation, application of methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylate (ACC) an immediate precursor of ethylene precursor could also induce systemic resistance against *P. syringae* DC3000 in NahG plants which cannot accumulate SA. MeJA cannot induce resistance in JA response mutant *jar1-1*, ethylene response mutant *etr1-1* and *npr1-1* whereas ACC could not induce resistance in *etr1-1* and *npr1-1* plants, but *jar1-1* mutants remained unaffected (Pieterse et al., 1998). These results revealed that PGPR mediated ISR response signaling pathway involves successive activation of JA and ethylene components to induce defense response and ISR is regulated by NPR1.

Ethylene insensitive mutants *etr1* are having insensitivity to ethylene only in root region, but not in shoots, so that when treated with WCS417 in root region they could not show induced resistant state but when bacteria were unfiltered in to the leaves they exhibited ISR response (Pieterse et al., 2000). The results indicated that ISR induction in *Arabidopsis* by WCS417 depends on the ethylene sensitivity at the site of application of PGPR. Clearly, complete ethylene signaling pathway is necessary for the manifestation of ISR. If ethylene signaling was requisite only for systemic expression of ISR at the site of pathogen infection, then *etr1-1* plants would be likely to produce usual levels of ISR in leaves on subsequent application of WCS417r to roots which suggest that ethylene is involved in induction or translocation of systemically transported ISR signal. Wild type *Arabidopsis* plants showed ISR response upon application of MeJA or ACC. *Jar1* mutant, respond positively to ACC application showing that responsiveness to ethylene is mandatory for inducing ISR after JA dependent signaling steps. On the other hand, MeJA application couldn't induce ISR in the *etr1* mutant showing JA and ethylene responsiveness is necessary for inducing ISR. MeJA and ACC both failed to induce ISR in NPR1 mutant indicating that both JA and ethylene needed upstream of NPR1 in ISR signaling cascade.

The theory of necessity of both JA and ethylene responsiveness for induction of ISR do not hold true for all the pathogen-plant-PGPR interactions. Certain exceptions in the case include ethylene responsive mutants *etr1-1* and *ein2-1* induce ISR against *Peronospora parasitica* (Iavicoli et al., 2003). ISR stimulated by *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 via volatile 2,3-butanediol do not require JA signaling. Such studies indicated that ISR response is generally not dependent on SA but also having different requirement for JA and ethylene signaling (Ryu et al., 2004).

10.3.2 Beneficial microbe mediated SA dependent ISR pathway

It is proven that ISR is activated by beneficial microorganisms and it is not dependent on SA signaling. But, few of the PGPR strains were reported to trigger SA dependent ISR pathway which is analogous to pathogen triggered SAR. Generally, it was established that plant beneficial microorganisms induce ISR pathway which is regulated by SA-independent mechanisms, but few of the PGPR strains are reported to induce SA-dependent type of ISR which is very similar to pathogen induced SAR. For example, *P. aeruginosa* 7NSK2 mutants producing SA increased disease resistance wild type bean and tomato plants but failed to induce disease resistance in SA non accumulating tomato plants (De Meyer and Höfte, 1997; Audenaert et al., 2002). SA overproducing mutant of *P. fluorescens* P3 was found to induce SAR response in plants. The mechanism behind SA producing PGPR mediated systemic resistance was believed to be the fact that SA produced by PGPR strains generally gets incorporated within SA moiety containing siderophores which increase ferric iron (Fe^{3+}) uptake and thereby making SA unavailable for triggering the SAR pathway. Same way, role of SA in ISR triggered by several *Trichoderma* strains was also proven (Contreras-Cornejo et al., 2011; Mathys et al., 2012; Martínez-Medina et al., 2013). Reactive oxygen species accumulated at the spot of the

establishment of PGPR strains are important elicitors for SA dependent SAR pathway induced by PGPR strains. Some of the cases of ISR showed exceptional involvement of SA in ISR induction in plants. Maurhofer et al. (1994) reported that induction of resistance in tobacco plants by *P. fluorescens* CHA0 was correlated with production of SA. Treatment of tobacco roots with *P. fluorescens* CHA0 enhances amount of SA-inducible PR-proteins in tobacco leaves. When SA biosynthesis genes were introduced into *P. fluorescens* P3 from *P. fluorescens* CHA0, the transformed strain shows increased resistance induction. De Meyer and Höfte (1997) reported that SA impaired mutants of *P. aeruginosa* TNSK2 could not induce resistance in bean and tobacco plants. Moreover, it could not induce resistance against tobacco mosaic virus in NahG tobacco plants.

10.4 Decoding genetic cascade of ISR response

It was established that in *Arabidopsis*, JA and ET signaling will activate specific defence related gene expression, but their exogenous application induce systemic resistance. Van Wees et al. (1999) evaluated alteration in expression of JA and ET responsive, defence linked genes viz. LOX1, LOX2, VSP2, PDF1.2, HEL, CHI-B and PAL1 in *Arabidopsis* plants showing WCS417r facilitated ISR. All the genes were not found to be up regulated in ISR expressing plants suggesting that resistance achieved was not linked to the significant increase in either JA or ET concentration and there was no change in production of any of the signaling components which proves that ISR is related to enhanced sensitivity to signaling molecules not to their higher production (Pieterse et al., 2000, 2001). To find out ISR linked genes expression pattern, Verhagen et al. (2004) monitored transcriptional profiling of 8000 *Arabidopsis* genes during WCS417r induced ISR. None of the gene out of 8000 genes studies displayed stable alteration in expression pattern in leaves due to root colonization by WCS417r, which indicates the fact that ISR response does not involve alterations in gene expression. Moreover, upon challenge inoculation with bacterial leaf pathogen Pst DC3000, total 81 genes displayed amplified expression pattern in ISR-expressing leaves which proves that ISR expressing plants are primed to react quicker and intensely during pathogen attack. Priming associated responsive genes were believed to be controlled by JA and ET signals that approve previous discoveries wherein *Arabidopsis* root colonization by WCS417r increase expression of JA and ET responsive genes VSP2, PDF1.2 and HEL (Van Wees et al., 1999; Hase et al., 2003; Van Oosten et al., 2008). Due to priming plants get increased ability for more quick and efficient activation of cellular defence responses upon pathogen infection Priming state is generally expressed at transcriptional level and ISR related transcriptional factors remains inactive under pathogen free conditions and accelerate defence response upon infection by pathogens. In *Arabidopsis*, transcription factor genes associated with AP2/ERF family were over represented. Pozo et al. (2008) revealed that promoters of JA responsive *Arabidopsis* genes expressed during ISR are considerable enriched with *cis*-acting G-box-like motif which is binding site for key transcriptional regulator of JA reliant defences i.e. MYC2 (Memelink et al., 2009). The *Arabidopsis* mutants *jin1* compromised for MYC2 cannot induce *P. fluorescens* WCS417r or *P. indica* facilitated ISR, which proved central role of MYC2 as transcriptional regulator for priming in ISR.

10.4.1 Root specific regulator for ISR

It has been proved that *Arabidopsis* mutant *eir1* having non sensitivity to ethylene in roots cannot elicit ISR response in leaves which proves the fact that ethylene signaling is crucial in root region for elicitation of ISR response in systemic tissues. ISR elicitation by PGPR comprise of long distance signaling which starts from root-microbe interface. Meager efforts were done to find out root specific signaling events and components involved in initiation of ISR. Moreover, R2R3 type MYB transcription factor gene *MYB72* was recognized to be primary part in elicitation of ISR in *Arabidopsis* roots due to colonization by *P. fluorescens* WCS417r (Verhagen et al., 2004). Minor expression of *MYB72* was monitored in vascular bundle of un-induced plants, whereas the gene remains greatly expressed in root epidermis and cortical cells of induced plants. *MYB72* knockout mutants of *Arabidopsis* induced with *P. fluorescens* WCS417r or *P. putida* WCS358r failed to express ISR in response to numerous foliar pathogens proving necessity of root specific transcription factor for elicitation of ISR (Van der Ent et al., 2008; Zamioudis et al., 2014). *MYB72* gene also expressed in *Trichoderma* mediated ISR in *Arabidopsis* roots and found to be critical (Pieterse et al., 2014). Overexpression of *MYB72* could not provide enhanced resistance to foliar pathogens, proposing that *MYB72* performs in combination with other signaling components. *MYB72* is precisely triggered in roots under iron-limited circumstances or conditions that alter iron utilization, like high zinc concentrations, directing to a connection between iron homeostasis and commencement of ISR (Palmer et al., 2013). Recognition of beneficial rhizosphere colonizing microorganisms triggers activation of transcription factor gene *MYB72* locally in the roots. Consequently, *MYB72* assumed to interact with

transcription factor EIL3. Downstream of, or in parallel with MYB72/EIL3, an unidentified ethylene signaling element is necessary in roots for commencement of ISR in leaves. The ISR signal transduction cascade needs NPR1, possibly in systemic tissue. Systemically, induction of ISR is accompanying with priming for improved expression of a set of JA-responsive and/or ET-responsive genes at the site of attempted pathogen entry. The pathogen attack activates resistance responses in plants that are speeded in ISR-primed plants. Van der Ent et al. (2008) reported gene MYB72 is specifically activated in the roots upon colonization by WCS417r. Moreover, T-DNA knockout mutants *myb72-1* and *myb72-2* are not able to support ISR against the pathogens *P. syringae* pv tomato. Yeast two-hybrid analysis shown that MYB72 substantially interacts *in vitro* with ETHYLENE INSENSITIVE3 (EIN3) LIKE3 transcription factor EIL3, connecting MYB72 task to ethylene response pathway. Nonetheless, WCS417r triggered MYB72 in ISR deficient, ethylene insensitive *ein2-1* plants. Furthermore, external application of the ethylene precursor ACC induced resistance in *myb72-1* to the wild type levels, proving that MYB72 acts upstream of ethylene in the ISR pathway.

10.4.2 Role of WRKY in ISR

WRKY proteins are emerging players in plant signaling and have been thoroughly reported to play important roles in plants under biotic stress like pathogen attack. Jiang et al. (2016) explained the mechanism through which inoculation of single PGPR strain stimulates ISR response by concurrently triggering both SA and JA/ET signaling pathways. They demonstrated that application of *Bacillus cereus* AR156 significantly stimulated the transcription of WRKY70, but blocked that of WRKY11 in *Arabidopsis* leaves (Jiang et al. 2016). Moreover, both WRKY70 and WRKY11 were found to be involved in AR156 mediated induced cellular defence responses and also transcription level of plant defence response genes. Overexpression both WRKY70 and WRKY11 transcription factors in *Arabidopsis* also demonstrated their essentiality for AR156 mediated ISR whereas AR156 triggered ISR was entirely stopped in double mutant of two transcription factors. ISR pathway was found to be partly retained in single mutants of either WRKY70 or WRKY11 transcription factor, which specifies that regulation of two transcription factors rely on two diverse pathways. Moreover, target genes of the two transcription factors and epistasis analysis proved that WRKY11 regulated AR156 mediated ISR by activating JA signaling pathway, whereas WRKY70 regulated ISR by means of activating the SA signaling pathway and both WRKY11 as well as WRKY70 modulated AR156 triggered ISR in NPR1 dependent mode (Niu et al., 2011).

WRKY transcription factors are involved in crosstalk amongst SA/JA pathways of defence. Generally, WRKY70, WRKY11, WRKY17, WRKY50, WRKY51, and WRKY62 were found to be involved in SA and JA pathway crosstalk (Pieterse et al., 2012). Li et al. (2004) revealed that overexpression of WRKY70, showed increased expression of SA responsive PR genes but alongside repressed expression of JA responsive marker gene *PDF1.2* in an NPR1 dependent mode, specifying the fact that WRKY70 acts as a positive regulator of SA induced genes and a repressor of JA responsive genes. An R2R3 MYB transcription factor of *Arabidopsis*, AtMYB44, controls antagonistic communication between SA and JA signaling via direct transcriptional control of WRKY70 (Shim et al., 2013). It was also established that WRKY50 and WRKY51 proteins facilitated SA suppression of JA signaling, exogenous application of SA inhibited JA inducible *PDF1.2* expression in wild type but not in *wrky50* or *wrky51* mutant plants (Gao et al., 2011). WRKY53 and HSPRO2 are positive regulators of basal resistance that acts downstream of SA and negatively controlled by JA and ET signaling (Murray et al., 2007).

10.5 Cross talk amongst ISR and SAR

Salicylic acid, Jasmonic acid and Ethylene are three key signaling components of induced defence pathway in plants. There exists cross talk amongst ISR and SAR pathway as JA and ET acts in combination for ISR response whereas SA can suppress JA dependent responses. Pharmacological and genetic analysis revealed that SA based suppression of JA inducible gene expression play a key role in this system. In some cases suppression of SA reactions by JA was observed. NPR1 serves as common regulator for both SAR and ISR pathways. The antagonistic effect of SA on JA signaling was proved to be regulated by NPR1. For expression of PR genes during SA based SAR response essentially requires nuclear placement of NPR1 and not needed to suppress JA signaling. Thereby crosstalk amongst SA and JA is controlled by unique function of NPR1 in cytosol. Mechanism of action of NPR1 in the cytosol is not known, but it is alluring to guess that it affects SCFCO11 ubiquitin-ligase complex that control JA responsive gene expression via directed ubiquitination and succeeding proteasome facilitated degradation of a negative regulator of JA signaling. Moreover, *Arabidopsis* transcription factor WRKY70 was also found to be involved in pathway cross talk, it can activate SA responsive genes and repress JA inducible genes, proving involvement of WRKY70 in integration of signals

from both antagonistic pathways (Li et al., 2004). Moreover, certain transcription factors like ERF1 and MYC2 can mix signals of JA and ET pathway for activation of defence associated genes reactive to both JA and ET. Such pathway cross talk provides benefit to the plants due to their great regulatory potential that assists plants to choose appropriate defensive mechanism based on type of pathogens encountered.

Van Wees et al. (1997) revealed relations amongst ISR and SAR pathway and proved that concurrent activation of both pathways provided additive effect for protection against Pst DC3000. *Arabidopsis* mutants impaired in either SAR or ISR, cumulative effect was not seen. Furthermore, expression of the SAR marker gene PR-1 was not changed in ISR and SAR showing plants, indicating that compatibility of both the pathways to act together. Furthermore, constitutive level of NPR1 is sufficient for simultaneous expression of both ISR and SAR pathways and do not require increase in concentration of NPR1 transcripts. SA response requires nuclear placement of NPR1. SA assisted repression of JA response do not rely on placement of NPR1 which shows that cytosolic NPR1 is able to suppress JA pathway. (Spoel et al., 2003) Additionally, nuclear NPR1 is essential for manifestation of transcription (co) factors that suppress JA mediated gene expression such as glutaredoxin 480 (GRX480), TGAs and WRKYs (Van der Does et al., 2013; Schmiesing et al., 2016), also suggestive of suppression of JA response also in a nuclear NPR1 reliant manner. GRX480 acts in SA and JA crosstalk, by suppressing JA-mediated response, in an NPR1 dependent manner (Ndamukong et al., 2007).

It is also evident that time and quantity of ethylene production also plays important role for crosstalk between the two defence pathways. Leon-Reyes et al. (2009) demonstrated proved that concurrent stimulation of ethylene and Jasmonic acid pathways in *Arabidopsis* intensely repressed NPR1 reliance of SA and JA crosstalk. Moreover, complete activation of ET and JA signaling (e.g. during infection with a necrotrophic pathogen) caused insensitivity to upcoming SA mediated suppression of JA/ET-dependent defences. In this way, ET assists plants to select JA/ET pathway over SA pathway in case of infection by multiple pathogens (Leon-Reyes et al., 2010).

Besides NPR1, other SA controlled signaling constituents appeared as key regulators of crosstalk between SA and JA/ET pathways. In *Arabidopsis*, TGA transcription factors are principally recognized as controllers of SA facilitated transcription, but they regulate activity of ET/JA reliant ERF branch as well. TGA2, TGA5, and TGA6 are mandatory for *B. cinerea* mediated expression of PDF1.2 to provide resistance (Zander et al., 2010). Conversely, in presence of SA, the similar TGAs have a part in SA facilitated antagonism of ERF branch signaling. The TGAs can straightway target activation sequence-1 (as-1) promoter sequence in the ERF transcription factor gene ORA59, and so regulating its expression (Zander et al., 2014). Along with observation displaying effect of SA on ORA59 protein accumulation (Van der Does et al., 2013), it also showed that ORA59 plays a principal role in crosstalk amongst SA and JA/ET pathways.

10.6 Conclusions

Plant defence system plays a key role for the existence of plants. It can be activated to fight with pathogens and shut down to accommodate mutualists. The primary signaling occurs at the site of root colonization by beneficial microorganisms. The first step for plant immunization with PGPR decoded but the signaling pathway underlying ISR starting from root still needs to be decoded. The issue of concern includes profiling of signal transduction molecules being activated by ISR eliciting microorganisms which further be translated to enhance defence response against foliar pathogens. MYB72 is needed for starting ISR from the roots. We are still in sort of knowledge regarding root associated factors responsible for ISR and how plant roots manipulate root microbiome for getting maximum benefit in the form of plant defence responses. Discoveries regarding plant metabolic profiling during ISR response will provide new in insight about genes involved and expressed during ISR response and further manipulation of such genes may be possible to get highly immunized plant system for future organic agricultural system.

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Chapter 11

Role of fungal elicitors in plant defense mechanism

Zalak M. Patel, Rita Mahapatra and Siva Satya Mohan Jampala

Post-Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Anand, Gujarat, India

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11.1 Introduction

Nowadays, a lot of attention is given to physiological and biochemical reprogramming of plants, triggered by various biotic and abiotic environmental factors which activates immunity of plant, called induced resistance (Nawrocka and Małolepsza, 2013). Plants have an extensive repertoire of responses by which they are able to localize, inhibit and isolate infections. The understanding of how plants identify a foreign microbe as a pathogen and how they interact with each other, is beneficial for crop improvement. For these reasons, much efforts have been invested towards understanding disease resistance mechanisms in plants (McDowell and Woffenden, 2003). In contrast to animals, plants being sessile, lack a circulating immune system to protect themselves against an array of pathogens with which they interact in their environment. Though plants are in continuous contact with a diverse microorganisms in the environment, relatively small proportion of pathogens are able to cause disease (Baker et al., 1997). Disease occurs when host is unable to check the entry of pathogen; when pathogen bypass the host active defense system by secreting metabolic toxins; and when the environmental condition is favorable for pathogen and unfavorable for the host (Surico, 2013; Narayanasamy, 2011).

Several factors like presence or absence of recognition factors, host receptors and essential nutrients for growth, determine the ability of any pathogen to cause disease (Guest and Brown, 1980; Anderson et al., 2010). The cell to cell communication is necessary for host and pathogen to initiate infection (Boyle and Finlay, 2003). The absence of specific recognition factors on host plant surface cells make difficult for the pathogen to adhere on host surface thereby preventing infection (Van Baarlen et al., 2007). For successful infection, pathogen must pass through the following processes; 1) the suitable host and the proper entry, 2) overcoming preformed and induced host defense systems and 3) absorption of nutrients and colonization of the host tissue. Once inside the host, pathogens proliferate and absorb the

host plant nutrients by disturbing normal physiological and biochemical processes of plants and causing disease (Hou et al., 2011; Zimaro et al., 2011). Here, this chapter will focus on the mechanism of systemic acquired resistance (SAR), a type of induced plant defense and the role of fungi derived elicitor for triggering SAR in plants by various studies such as determination of effective concentration of elicitor for induction of SAR, *in vitro* and seed treatment study, pot experiments, field study as well as in screening of resistant variety.

11.2 Types of defense mechanism

Pathogens have developed sophisticated weapons to attack plants while on the other side plants also possess established and diversified strategies to defend against pathogens. Plants possess an innate immunity comprising several structural, chemical, and protein-based defenses intended to detect and stop invading pathogens. The pre-formed or constitutive (passive) as well as inducible (active) defense systems of plants help to protect themselves against attack from the vast array of viruses, bacteria, fungi, nematodes and insects (Hammond-kosack and Jones, 1997; Montesano et al., 2003).

11.2.1 Pre-formed or constitutive defense

The pre-formed or passive resistance is the basal first line of immune defense and involves physical and chemical factors present in plants prior to the pathogen attack. These include plant's surface structures such as topography of leaf surface, thickness of the cuticle, conformation of cuticular wax and degree of stomatal opening etc. A thick cuticle repels airborne propagules and prevents the direct penetration of the pathogen into the host epidermal cells (Dangl and Jones, 2001). Waxes on leaf and fruit form a water repellent surface thereby prevents the retention of water on which pathogen spores can germinate (Nurnberger and Lipka, 2005), penetration by pathogens difficult or impossible. Similarly, pore size and closing of stomata is highly regulated by plants in response to pathogen attack (Jones and Dangl, 2006). In addition, plants also secrete a variety of biochemical substances having inhibitory action against certain pathogens. Essential oils like thyme, cinnamon, sage and savory often protects against pathogen attack (Guest and Brown, 1980). Phenolic compounds, terpenoids, tannins and dienes which are present in high concentrations in fruits, leaves or seeds are potent inhibitors of hydrolytic or proteolytic macerating enzymes of plant pathogens. Several other types of pre-formed compounds such as avenacin (saponin) have detergent like properties which disrupts the fungal cell wall (Doughari, 2015) and also eliminate fungal pathogens lacking enzymes for breaking down saponin (Osborn, 1996).

11.2.2 Induced (active) defense

Induced or active resistance is categorized into two types: induced systemic resistance (ISR) and systemic acquired resistance (SAR). Induced systemic resistance (ISR) is activated by the non-pathogenic rhizobacteria or antagonistic fungi and does not involve salicylic acid but depends on the jasmonic acid (JA) and ethylene signaling pathway (Van Loon et al., 1998). Systemic acquired resistance (SAR) is induced in response to infection by necrotizing pathogen (Ryals et al., 1996) and confers long-lasting protection against a broad spectrum of microorganisms (Durrant and Dong, 2004). In SAR, the resistance capacity is amplified in the primary infected parts as well as distantly located non-infected tissue hence denoted as systemic acquired resistance (Kothari and Patel, 2004).

Plants have induced resistance that prevent further colonization of the tissues by the pathogen if the structural barrier is broken by the invading pathogen. Induced or active defense comprises substances are either absent or present in negligible amount before infection and are synthesized or activated after infection. The lignification makes the cell walls highly impermeable for pathogen (Guest and Brown, 1980). Sometimes, the infected cells are surrounded by suberized cells to isolate the infection site from healthy tissue (Van Baarlen et al., 2007). Abscission layer may be formed to drop-off the invaded plant parts along with pathogen. The gums and vascular gels quickly accumulate within the cell surrounding the infection thread and haustoria to make it starve and die (Doughari, 2015). The tyloses are formed to block the spread of pathogenic strains (Jan et al., 2011; Dou and Zhou, 2012). The synthesis of secondary cell wall and papillae formation, callose deposition may be also effective against slow growing pathogens or weak parasites (Anderson et al., 2010). On the other hand, phenolic compounds like chlorogenic acid, caffeic acid, hydroquinone and phytoalexins are toxic and inhibit the growth of the pathogens (Jones and Dangl, 2006). Plants generally respond to the biotic or abiotic stress by the production of phytoalexins and these antimicrobial compounds are synthesized by the cells surrounding the infection or by the infected cells making toxic environment for the invading pathogen and leading to the hypersensitive cell death (Jones and Dangl, 2006; Cai et al., 2009).

However, in contrast to the phytochemicals, production of defensive proteins requires a large amount of plant resources and energy, therefore, they are only produced in significant quantities after a pathogen attack. But after the activation, defense proteins and enzymes efficiently inhibit microbial pathogen. Some of the proteins and enzymes include lectins, defensins, ricin, protease inhibitors, amylase inhibitors, hydrolytic enzymes, glucanases and chitinases, lysozymes and hydroxyproline-rich glycoproteins (Jan et al., 2011; Dou and Zhou, 2012). Defensins display a wide range of biological activities that aid to inhibit the growth of many fungi and bacteria (Guest and Brown, 1980; Buonauro, 2008). Hydrolytic enzymes produced in response to pathogens often accumulate in extracellular spaces to degrade the cell walls of pathogenic fungi (Freeman and Beattie, 2008; Piña-Vázquez et al., 2012). Chitinases degrade the chitin, present in the fungal cell walls. Similarly, glucanases catalyse the degradation of glycosidic linkages in glucans, a biopolymer, present in the cell walls of many oomycetes. Lysozymes are hydrolytic enzymes degrading bacterial cell walls. *In vitro* analysis has proved the anti-fungal nature of these compounds and transgenic plants expressing high content of these enzymes display amplified resistance to a broad range of pathogens (Freeman and Beattie, 2008).

11.3 Mechanism of plant pathogen interaction

Plant pathogen interaction normally follows the recognition of pathogen or the elicitor molecule present on fungal cell by specific receptor present in plant which initiate signal transduction to activate plant defense mechanism. The binding of elicitor receptor molecule, leads to the increased ion fluxes, rapid changes in protein phosphorylation, accumulation of pathogenesis related (PR) proteins, hypersensitive response (HR), synthesis of signaling molecules such as salicylic acid, antimicrobial compounds and secondary metabolites. The cumulative effect of all these results initially into local and delayed systemic resistance in whole plant as discussed here.

11.3.1 Pathogen recognition in plants

To efficiently control conquering pathogens, plants must distinguish them as foreign components. Therefore, plants have evolved well-organized signaling mechanisms to detect microbial pathogens. Recognition of pathogen often starts following the perception of intruding microbes or their products by microbe or pathogen associated molecular patterns (MAMPs or PAMPs). Pattern Recognition Receptors (PRRs) are certain class of plasma membrane bound extracellular receptors which are capable of detecting or recognizing microbial elicitors. In response to MAMPs, activated PRRs leads to active defense responses against the pathogens which inhibit the pathogen growth before they could cause any serious infection in the plant (Zipfel and Felix, 2005). Examples of such as chitin, oligogalacturonides, ergosterol, xylanase, cold-shock protein, bacterial flagellin and lipopolysaccharides (LPS). Pathogen recognition is a non-specific process denoted as PAMP triggered immunity (PTI) and such non-self recognition by plants is generated by the help of elicitors (Mogensen, 2009).

11.3.2 Elicitor molecules

Elicitors are the low molecular weight compounds which triggers plant immune response by activating signal cascade. Elicitors are classified into two types i.e. pathogen derived elicitors (exogenous elicitor) and plant derived elicitors (endogenous elicitor). Most of the exogenous elicitors of the plant defense responses are nonspecific and differ widely in their chemical nature including protein, oligosaccharides, glycoprotein and lipids. Inducible defense responses can be triggered, not only upon the encounter of the plant tissue by pathogen, but also upon the elicitor treatment. Therefore, elicitors are now extensively used to study the molecular mechanism of defense responses (Suzuki, 1999).

Abiotic elicitors like heavy metal ions, UV light and some metabolic inhibitors, induce physiological stress responses and contribute to resistance. Their effect is generally temporary and non-specific (Guest and Brown, 1980; Jones and Dangl, 2006; Buonauro, 2008; Freeman and Beattie, 2008). Biotic elicitors are specific in targeted actions which comprise secondary metabolites with more lethal effects. These specific elicitors comprise Damage Associated Molecular Patterns (DAMPs). Many fungal and bacterial pathogens produce lytic enzymes like polygalacturonase, cell wall fragments and peptides which block or destroy the mechanical barriers such as the middle lamella of plant tissues. These DAMPs function as endogenous elicitors and typically appear in the apoplast as danger signals to induce innate immunity (Jones and Dangl, 2006; Cai et al., 2009). Elicitins are a group of similar peptides from related oomycetes e.g. *Phytophthora* sp. Polyunsaturated fatty acids i.e. arachidonic and eicosapentaenoic acid from cell membranes of *Phytophthora* sp. provoke defense response in potatoes. Elicitors produced by avirulence genes in the pathogen are known as Gene-specific elicitors (Doughari, 2015). Here, the recognition of a pathogen takes place when product of

avirulence (Avr) gene, secreted by pathogen interact with the product of resistance (R) gene in plant which is known as gene for gene model. A complementary pair of genes (gene-for-gene) present in a particular pathogen race and a host cultivar determines this cultivar specific resistance.

11.3.3 The 'gene-for-gene' hypothesis

Presence of the 'R' gene and corresponding 'Avr' genes lead to recognition which activate resistance of the plant and avirulence of the pathogen. The compatible reaction occurs when 'Avr' gene in the pathogen or 'R' gene in the host is absent or is mutated, resulting in disease (Nurnberger and Sheel, 2001). As a result of binding, a signal transduction cascade is activated leading to the activation of a variety of plant defense responses. In the host, the genes for resistance are mostly dominant over that for susceptibility. While on the other hand, the genes for avirulence are usually dominant over that for virulence in the pathogen. The process of recognition of the pathogen avr gene product (elicitor) by the host R gene product (receptor molecule) is called incompatible plant-pathogen interaction. Mutation in avr gene of a pathogen evades gene-for-gene recognition by the host R genes and thereby breaking down the host resistance. However, a new variants of R gene also evolve through survival pressure and selection that codes a modified receptor which can distinguish the modified avr gene product. Likewise, a number of diverse R genes have evolved in a host plant to corresponding virulence genes of the pathogen (Heath, 2000). All the R proteins contain a common motif of leucine rich repeat (LRR) and some R proteins contain nucleotide binding sites (NBS) and or leucine zipper (Grover and Gowthaman, 2003). LRRs are believed to mediate protein-protein interactions or determine specific recognition of ligands by the receptor molecules (Kobe and Deisenhofer, 1994). All the receptors are highly specific for pathogenic strains and individual R genes have narrow recognition abilities (Odjakova and Hadjiivanova, 2001). Thus, specific recognition of an aggressor by a plant requires the presence of matching avr and R genes respectively in them.

11.3.4 Alkalinization of medium

Most of the defense responses involves ionic change, synthesis of secondary metabolites and development of physical barriers against infections (Pusztahelyi et al., 2015). The initial defense response provoked in cell cultures against MAMPs is the alkalisation of the growth medium i.e. changes in calcium, proton, potassium and chloride ions in the plasma-membrane. Elevation of calcium ion in cytosol plays a crucial role in controlling production of reactive oxygen species (ROS), salicylic acid (SA) and stomatal closure (Dou and Zhou, 2012).

11.3.5 Nitrosative and oxidative burst

The production of reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROS) are the initial reactions for the activation of plant defense. The role of ROS has been well studied but for RNI remain mostly unclear. Previous reports show that there is a covalent linkage between the nitric oxide (NO) and cysteine thiol to form an S-nitrosothiol in protein which is a redox-based post-translational modification through a cascade of biochemical pathways to produce RNI (Jan et al., 2011; Dou and Zhou, 2012). The NO directed negative feedback loop is generated by S-nitrosylation of the NADPH oxidase (at Cys 890) at higher concentrations of S-nitroso thiols which inhibits the synthesis of ROS thereby restricts the HR.

ROS are potential toxic equivalent to reduced oxygen forms such as hydrogen peroxide, superoxide anion and hydroxyl free radical (Somssich and Hahlbrock, 1998). Normal cells appear to have a balance between them to escape from cytotoxicity by the action of catalase, peroxidase and superoxide dismutase. In susceptible varieties, the scavengers are highly active which prevent the oxidative burst and the resulting fungal toxicity (Gonner and Schlosser, 1993). They play important role like antimicrobial activity, oxidative cross-linking of glycoproteins for cell wall strengthening (Scheel, 1998), triggering of intracellular signaling pathway i.e. the synthesis of salicylic acid (SA) (Piffanelli et al., 1999) and activation of systemic acquired resistance associated with systemic proliferation of the oxidative burst (Piña-Vázquez et al., 2012; Gomathi et al., 2011). The changes in the ion flux and oxidative burst are crucial for a complex and highly integrated signaling network. The local accumulation of reactive oxygen species and nitric oxide can act as secondary messengers for hypersensitive response and expression of defense genes.

11.3.6 Effector triggered immunity (ETI)

Effector triggered immunity (ETI) is the plant immune response that specifically include transcription processes in the host nucleus. For example, successful pathogens endorse pathogenesis by injecting effector proteins into the plant cell. These Avr proteins which are avirulent in nature contributes to pathogen virulence in three ways: by acting as transcription factors, by disturbing histone packing and chromatin configuration, by directly targeting activity of transcription factor and eventually releasing nutrients for the survival of pathogen (Piña-Vázquez et al., 2012; Surico, 2013). Plant ETI therefore respond to pathogen infection by activation of transcription factors including a range of pathogenesis related genes in and around the invaded plant cell for the synthesis of chemical substances such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA), various antimicrobial compounds as well as lignification and strengthening of cell-wall. Thus accumulated salicylic acid in the infected areas binds to the receptor NPR3 of the PR genes for the degradation of the infected cell by a process analogous to the apoptosis- a programmed cell death found in animals (Jan et al., 2011; Dou and Zhou, 2012).

11.3.7 Hypersensitive response (HR)

The oxidative burst is an immediate effect of a pathogen invasion into the plant that results in cytotoxicity causing hypersensitive response, which prevents further development of pathogen in the host tissue (Keppler et al., 1989). Accumulation of salicylic acid is followed by the activation of hypersensitive response thereby restricting the growth of pathogens and this necrotic response gives broad spectrum resistance against various plant pathogens (Mandadi and Scholthof, 2013). However, HR involves a complex series of reactions acting synergistically or additively. Cells surrounding the necrotic lesions become stubborn to subsequent infection, referred to as localized acquired resistance (Baker et al., 1997). Localized acquired resistance (LAR) often triggers non-specific resistance to the whole plant. After a previous and local interaction with a pathogen, the whole plant becomes resistant to the subsequent challenge of infection by broad range of pathogens. This long lasting non-specific defense is known as systemic acquired resistance (SAR) (Ryals et al., 1996). Initiation of the multi-component defense reactions at local level results in the quick development of local resistance and delayed systemic resistance (Scheel, 1998). Plant tissues may become highly resistant to the subsequent infection by a broad range of pathogens for an extended period of time once HR has been triggered. This systemic acquired resistance (SAR) represents a heightened state of readiness for subsequent infection (Freeman and Beattie, 2008). HR subsequently leads to programmed cell death (PCD) characterized by cytoplasmic shrinkage, mitochondrial swelling, chromatin condensation, vacuolization and chloroplast disruption (Dou and Zhou, 2012).

11.4 Role of fungi derived elicitors

Elicitors of fungal origin play various role upon application on plant. These can be used to enhance synthesis of commercially important secondary metabolites. Elicitor treatment changes the physiological conditions in plants to protect themselves from various biotic and abiotic stresses such as pathogen attacks, wounds, heavy metals, air pollutants, ultraviolet rays and harsh environmental conditions. Nowadays several researchers tried to understand the effect of elicitor treatment on plants by induction of defense related enzymes activity upon various mode of treatments i.e. effect on seed germination, screening of resistant variety, plant immunization under *in vitro* condition, pot and field studies. The treatment time and concentration of elicitor plays significant role in induction of plant disease resistance. An elicitor treatment is presumed to generate signals that activate the nuclear genes involved in the plant defense response leading to induction of defense related enzymes. In addition, higher accumulation of phenols, phytoalexins and deposition of callose and lignin at pathogen invasion site contribute to multi-layered plant defense system.

11.4.1 Role of elicitors in screening of resistant variety

The biochemical basis of resistance in tissue culture banana plants of resistant cv. Goldfinger and susceptible cv. Williams to Fusarium wilt caused by the pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc) Race 4 was investigated by inoculating with conidial suspensions to evaluate the degree of tolerance between the clones (De Ascensao and Dubery, 2000). In this study, defense responses induced by treatment of the plants with an elicitor prepared from the mycelial cell walls of Foc showed induction of lignin deposition, phenolics, and increased activity of the enzymes for cell wall strengthening i.e. phenylalanine ammonia lyase, polyphenol oxidase and peroxidase and cinnamyl alcohol dehydrogenase. Root tissue of the Goldfinger (tolerant cv.) responded to the fungal elicitor through the high deposition

of lignin, led by the elicitation of the enzymes involved in the synthesis of lignin, whereas only minor increases were detected in the Williams (susceptible cv.). These indicate a significant role of elicitors in cell wall strengthening by deposition of lignin as an induced defense mechanism in banana roots against *F. oxysporum* f. sp. *cubense* Race 4. This could be a useful tool for early identification of *F. oxysporum* f. sp. *cubense* resistance banana clones.

The study conducted on resistant and susceptible hybrids of banana clones, noted the earlier and enhanced accumulation of defense enzymes involved in phenylpropanoid pathway i.e. phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), catalase, superoxide dismutase (SOD) and PR-proteins like β -1–3 glucanase and chitinase as well as phenols in banana against inoculation of *F. oxysporum* f. sp. *cubense* (Foc). Isozyme analysis revealed the induction of specific isoforms of peroxidase (PO) and polyphenol oxidase (PPO) in the resistant hybrids upon challenge inoculation (Kavino et al., 2009).

Further work on elicitor prepared from the *Fusarium oxysporum* f. sp. *cubense* (Foc) was shown to induce the activity of defense related enzymes i.e. peroxidase, polyphenol oxidase, β -1,3-glucanase, chitinase and phenolics in leaves of resistant and susceptible variety of banana. In susceptible variety, defense-related enzymes activity was increased to the level of untreated resistant variety indicating elicitor-induced defense in susceptible variety reached to the apparent level of untreated resistant variety. This elicitor-treated susceptible variety showed no symptoms upon fungal forced inoculation after 2 months depicting immunization or priming make the susceptible variety capable to tolerate further pathogen infection (Thakker et al., 2011). The amplified activity of β -1,3 glucanase in resistant and susceptible varieties of groundnut seeds after challenged inoculation with *A. flavus* indicate the elicitation of defence mechanism against *A. flavus* infection in peanut seed (Liang et al., 2005).

The use of culture filtrate of *Lasiodiplodia theobromae* increased respiration rate, phenylalanine ammonia lyase activity, hydrogen peroxide and lipid peroxide content in *Brassica nigra* plants and excess ROS generated in this particular plant-mycotoxins interaction, also causing necrosis at the local site of pathogenesis. Initially, salicylic acid (SA) content increased followed by decrease upon treatment with culture filtrate (Thakkar et al., 2004). In this study, SAR was reported to be constrained in *B. nigra* plants infected with *L. theobromae* due to the lack of SA, a key signaling molecule for SAR while exogenously supplied SA developed resistance as well as delayed plant death. The authors hypothesized that jasmonic acid produced by fungus inhibits SA biosynthesis thereby preventing the development of systemic resistance.

11.4.2 Role of fungal elicitors as vaccine for plant immunization

Fungal components i.e. fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) prepared from *Fusarium oxysporum* f. sp. *cubense* successfully induced SAR in *in-vitro* propagated banana plantlets by eliciting the defense enzymes activity along with signal molecules, salicylic acid (SA) and total phenol content. The results obtained in this study are important towards understanding the biochemical basis of fungal components given as elicitor (Patel et al., 2004). The treatment with elicitor prepared from the pathogenic fungi, *Fusarium oxysporum* f. sp. *cubense*, effectively induced PR-proteins and enzyme like β -1, 3-glucanase thereby increasing the resistance of banana plants against fusarial wilt (Thakker et al., 2009). A partially purified 100 kD β -1, 3-glucanase showed direct antifungal effect on the pathogenic strain. This may be one of the possible mechanisms of action induced in banana plants upon exposure to elicitor against Fusarial wilt. Therefore, elicitors could be used as plant vaccines for plant immunization.

The response of banana plant against dead and live pathogen of Fusarial wilt in terms of elicitation of defense-related enzyme activity i.e. β -1,3 glucanase, chitinase, peroxidase (POX), polyphenol oxidase (PPO), and phenolics was carried out (Thakker et al., 2013). The study revealed that interaction of banana plant with live pathogen resulted in early induction of defense. But, pathogen caused disease by overcoming the plant defense. Whereas, treatment with dead pathogen lead to escalation of defense responses in plants. Later on plants treated with dead pathogen showed resistance to even fungal forced inoculation of live pathogen. Thus dead pathogen was able to mount defense response in plants and provide resistance upon subsequent infection which shows that elicited plants are less susceptible to further pathogen attack.

Kuvalekar et al. (2011) noticed that alteration in the activity of peroxidase and polyphenol oxidase was not significant in healthy and *Uromyces hobsoni* Vize. infected stem tissues, while the leaves displayed a remarkable rise in the enzyme activity at advanced stage of disease. A noteworthy increase in the phenolic content was also detected at early and advanced stage of disease respectively in leaves and stem. These experiments correlate the predisposition of the host with the temporal activity of defense enzymes and the concentration of preformed antimicrobial compounds during the disease progression. Initial expression of oxidizing enzymes like POX and PPO results into the increased oxidation of polyphenols in the infected tissues making a biochemical barrier for pathogen for disease development. Higher

activity of POX was observed during *Uromyces vignae* and *U. fabae* infection in cowpea and broad bean respectively along with elicitation of various isozymes of POX in infested wheat plants (Mould et al., 2003; Jakupovic et al., 2006). Higher POX activity during an oxidative burst and hypersensitive response in the cells was also noticed in the resistance of coffee to orange rust *Hemileia vastatrix* (Silva et al., 2008). Gogoi et al. (2001) noticed an immediate increase in POX activity following inoculation of resistant wheat genotypes with *Neovossia indica* whereas susceptible genotypes showed a slow increase in POX activity.

Plant immunization against a broad spectrum of pathogens is an alternative strategy to the use of pesticides for disease control. Wu et al. (2013) performed an experiment in order to elucidate the mechanism of SAR to Fusarium wilt in Cavendish banana. Inoculation of the second leaf of banana plantlet with incompatible non-pathogenic *Fusarium oxysporum* f. sp. *cubense* race 1 triggered resistance in the roots to a succeeding challenge inoculation with virulent/ pathogenic Foc tropical race 4 in *in vitro* plantlets growing on modified medium without a carbon source (Wu et al., 2010). It is confirmed that the disease severity evaluated by *in vitro* pathosystem is not only consistent pot system in greenhouse (Ghag et al., 2012) but also in the field (Wu et al., 2010). The disease severity of the treated plantlets was similar to the resistant clones when the time period between primary infection and challenged inoculation was 10 days. The activities of defense enzymes like PAL, POX, PPO, SOD and SA content in roots attained the higher level than the control on 10 days after primary infection. The results of this study showed that induction of SAR by incompatible Foc race 1 is accompanied by the increase in the activities of defense related enzymes upon subsequent infection with pathogenic strain.

Upon the plant-pathogen interaction or elicitor treatment, salicylic acid (SA) accumulates for signal transduction which activates PAL, POX, β -1,3 glucanase and chitinase with increased production of secondary metabolites like phenols and phytoalexin and lignin content. Maize seeds treated with fungal culture filtrate of *Aspergillus flavus* and *A. paraciticus* and grown in respective fungal-infested soil showed 100% survival rate without any disease incidence by inducing systemic resistance in elicitor-treated plants against pathogen (Mahapatra et al., 2015). The application of both *A. flavus* and *A. paraciticus* FCF help the plant to synthesize the necessary components required for disease resistance by eliciting PAL, POX and β -1,3 glucanase enzymes which may lead to systemic resistance in maize plants (Mahapatra et al., 2015). It is assumed that the increased resistance in maize to *Aspergillus* ear rot after elicitor treatment may be related to accumulation of PR proteins particularly chitinase and β -1,3 glucanase. This study also showed increase in carbohydrate content after fungal elicitors treatment thus suggesting the higher metabolic activities required to combat the disease condition. This study demonstrates that the increase in yield along with number of seeds per cob in fungal elicitor treated plants may be associated with the stimulation of physiological processes i.e. higher protein, carbohydrate and chlorophyll content and nitrate reductase (NR) and nitrite reductase (NiR) activities along with improved vegetative growth of the plants (Mahapatra et al., 2015).

A noteworthy increase in POX activity was detected in pearl millet upon the treatment of elicitor derived from the cell wall of *Aspergillus niger* (Hindumathy et al., 2006). Non-host resistance was induced by elicitation of PAL enzyme activity in *Triticum aestivum* against *Sclerotinia sclerotiorum* (Basha, 2007). Treatment of potato tubers with elicitor derived from hyphal wall components of *Phytophthora infestans* elicited NR gene transcriptionally and protein level leading to NO production (Yamamoto et al., 2003).

Fungal mycelial cell wall extracts of *F. oxysporum* ssp. *lini*, *Botrytis cinerea* and *Phoma exigua* are effective elicitors for enhanced production of secondary metabolites in *H. perforatum* cell suspensions (Gadzovska Simic et al., 2015). Higher accumulation of phenylpropanoids and flavonoids can be correlated with higher activities of phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI) in fungal elicited cells. Moreover, elicitor treated *H. perforatum* cells synthesized higher content of naphthodianthrone representing an important part of defense strategy triggered by fungal elicitors. Antioxidant activity in fungal elicited cells remains significantly higher throughout the post-elicitation period, suggesting the accumulation of phenolic compounds that exhibit high antioxidant properties (Gadzovska Simic et al., 2015). For the effective elicitation, the treatment time and concentration of fungal extracts were standardized. The elicitors prepared from pathogenic fungi *A. panax* and *C. destructans* have enhanced accumulation of ginsenoside by adventitious root formation in *Panax quinquefolius* during AR bioreactor culture (Yu et al., 2016). In bioreactor culture, elicitors extracted from *A. panax* and *C. destructans* simulated adventitious root formation in *P. quinquefolius* for enhanced accumulation of ginsenoside (Yu et al., 2016). The highest content of nitric oxide (NO), putrescine (Put), and ginsenoside were detected at 2, 4, and 8 days after elicitation by both the fungal extracts respectively indicating NO and Put might be upstream signals for ginsenoside synthesis (Yu et al., 2016).

Treatment of rice seedlings with *Magnaporthe oryzae* hypersensitive protein 2 (MoHrip2) as an elicitor have shown an induced resistance to rice blast. MoHrip2-treated samples showed 10 newly induced proteins, 4 up-regulated and 3 down-regulated protein compared to control (Khan et al., 2016). These 17 differentially expressed proteins were found

to have different functions like defense-related proteins and transcriptional factors, reactive oxygen species (ROS) production, programmed cell death (PCD), signal transduction-related proteins and photosynthesis and energy-related proteins. The results demonstrated that MoHrip2 activated an early defense response in rice proved at the protein level (Khan et al., 2016).

After 8 weeks of co-culturing, mycelium extract (ME), protein polysaccharide fraction (PPF) and ethanol sediment (ES) prepared from four endophytic fungi, *Pestalotiopsis* sp., *Talaromyces* sp., *Hypoxylon* sp. and *Xylariaceae* sp. exhibited strong stimulation on biomass yields and active ingredients in their host *Dendrobium catenatum* (Zhu et al., 2018). Without any changes in the appearance of the plantlets, the crude extracts of fungal mycelia effectively promoted biomass and accumulation of active ingredients varying with elicitor origin and its chemical nature in *D. catenatum*. Moreover, PPF was found as the leading active constituent responsible for the enhancement of biomass, polysaccharides, naringenin and schaftoside as well as for the productions of total flavonoids and total phenols (Zhu et al., 2018). Treatment of *Cymbidium goeringii* seeds with mixed fungal elicitors increased the germination rate by 15–30% than that of untreated control whereas no significant change was observed in *C. faberi* treated with mixed fungal elicitors. Further mixed fungal elicitors also promoted acclimatization of tissue culture seedlings and also help new seedlings to survive upon transplantation (Gong et al., 2018).

The effect of Opera 183 g/L SE (Pyraclostobin + Epoxyconazole), a strobilurin group of fungicide derived from the extract of *Strobilurus tenacellus* was evaluated to control southern blight and collar rot of groundnut plant (Amin and Jampala, 2018). On detached groundnut leaves, distinct necrotic lesions were observed within a day after elicitor treatment indicating hypersensitive response. The initial study showed early seed germination along with increased root length in 0.15% Opera treatment than control. The earlier study also showed the Pyraclostobin (Headline) treated groundnut seeds with lower mortality rate and disease incidence compared to untreated seeds (Amin et al., 2015). Soil infestation study with *Sclerotium rolfsii* and *Aspergillus niger* revealed lower mortality rate and enhanced growth in 0.15% Opera treated plants than untreated seeds sown in pathogen infested soil. Furthermore, foliar treatments of Opera given on flowering stage and pegging and maturation stage, showed a significant increase in activities of defense-related enzymes like PAL, β -1,3-glucanase, POX, CAT, SOD, NO, growth and yield parameters such as weight of pods, number of seeds and test weight of seeds. This study supports the development of induced resistance by the elicitation of the phenylpropanoid pathway.

11.4.3 Determination of effective elicitor concentration

Elicitor concentration and time for the treatment is very important for induction of defense mechanism. Various concentrations and treatment time of elicitors were used by researchers to determine the effective elicitor concentration for seed and foliar treatment. The defense response in *Arachis hypogaea* was triggered using fungal components i.e. fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) of *Sclerotium rolfsii* (Nandini et al., 2010) and *Aspergillus flavus* (Nandini and Bariya, 2014) as elicitors. Based on the higher defense enzymes activity in elicitor treated *in vitro* grown groundnut plants, authors selected 10% concentration of 21 days old fungal culture filtrate (FCF) and mycelial cell wall fraction (MCW) of *Sclerotium rolfsii* (Nandini et al., 2010; Amin, 2017), *Aspergillus flavus* (Nandini and Bariya, 2014) and *Aspergillus parasiticus* (Nandini, 2011). Mahapatra et al. (2015) also found that 10% concentration of 21 days old FCF prepared from *A. flavus* and *A. parasiticus* induced increased activity of PAL, POX and β -1,3 glucanase in *in vitro* raised maize plants compared to other concentration used for standardization. On the other hand, Patel (2016) reported that treatment with 10% concentration of 15 days old FCF of *F. oxysporum* f. sp. *cumini* for 3 h, 5% concentration of 23 days old FCF of *A. burnsii* for 1 h, and 20% concentration of 24 days old FCF of *T. harzianum* for 24 h were most effective for induction of defense enzymes in cumin seeds.

11.4.4 Induction of HR by various fungal elicitors

Hypersensitive response (HR) is characterized by accumulation of antioxidative enzymes, synthesis of different phenolic compounds and phytoalexins. In this study, FCF-treated leaves showed the hypersensitive response, appearance of necrotic lesions which indicates the plant response to the pathogen-derived elicitors by synthesizing the defense enzymes thus protecting themselves against the pathogen. However, the appearance of hypersensitive response in MCW-treated plants is insignificant compared to FCF-treated plants (Nandini, 2011). Patel (2016) confirmed that *Fusarium oxysporum* f. sp. *cumini* and *Alternaria burnsii* derived fungal culture filtrate were found to contain 29 kDa glycoprotein as an active elicitor molecule which triggered immune response in cumin plant. Mahapatra (2014) revealed the presence of 14.3 kDa glycoprotein in column purified fraction of *A. flavus* and *A. parasiticus* derived FCF and

treatment with this purified protein also showed hypersensitive response on maize leaves along with induction of higher activity of PAL and POX. Nandini (2011) obtained 14.3 kDa and 20.1 kDa protein from purified culture filtrate of *S. rolfii* and *A. flavus* and *A. parasiticus* which indicated intense necrotic lesions on groundnut leaves upon treatment showing hypersensitive response. Amin (2017) also reported necrotic lesions induced by 21 days old fungal culture filtrate of *S. rolfii* upon treatment in groundnut leaves as well as found the presence of two isoforms (40 kDa and 38 kDa) of polygalacturonase (PG) in purified FCF prepared from *S. rolfii* as an active component.

11.4.5 Seed treatment study

Elicitors are thought to induce early seed germination by increasing salicylic acid synthesis which in turn leads to higher auxin production, a plant growth hormone, promoting enhanced radical formation. Patel (2016) carried out experiments to evaluate the effect of fungal elicitors on germination rate and vigor of cumin seeds in petriplate and pot and found highest germination rate in *Fusarium* derived elicitor treated cumin seeds followed by *Alternaria* derived elicitor and MIX FCF treatments. Cumin seeds treated with MIX FCF exhibited the increased radical length than other treatments. These elicitor treatments resulted in early emergence of radical compared to control seeds. Seeds of two maize varieties (Nitya-33 and Pioneer-30V92) germinated on MS media comprising *A. flavus* and *A. parasiticus* (AFFCF and APFCF) derived fungal culture filtrate showed elicitation of various defense related enzymes activity such as PAL, POX, β -1,3-glucanase, total protein and total phenols content than other elicitor treatments and control seedlings of both maize cultivars in 7 days old seedlings (Mahapatra et al., 2015). Amin (2017) also found that groundnut seed treatment with *S. rolfii* derived FCF for 24 hrs was significantly effective for early emergence of radical and plumule with higher germination rate than control.

11.4.6 Pot assay

It is hypothesized that elicitor treatment induces resistance mechanism in the seedlings and plants which ultimately enhance the production of defense enzymes, phytoalexins and other secondary metabolites which directly or indirectly acts upon pathogen by restricting their growth and infection. Patel (2016) reported lowest mortality rate (less than 20%) along with good germination and growth in *Fusarium* and *Alternaria* FCFs treated cumin seeds after 20 days of sowing in respective pathogen infested soil. These FCF treated plants reported higher induction of defense enzymes like PAL, POX, PPO, CAT, β -1, 3 glucanase, total protein and phenol content after 25 days of sowing. The subsequent experiment with foliar application of elicitor in 25 days old cumin plants also induced increased activity of defense enzymes and extra bands of induced protein and phenols than control plants. Various experiments carried out with single or double foliar application of elicitors at 25 days of interval in field grown cumin plants showed higher induction of various defense enzymes than control. *Fusarium* derived elicitor treated plants showed 10–14 days early flowering along with greener and healthy plants compared to control plants. Two maize cultivar seeds treated with fungal elicitors *A. flavus* and *A. parasiticus* FCF sown in *A. flavus* and *A. parasiticus* infested soil as challenged inoculation showed 80–100% survival rate with no sign of disease incidence and higher induction of various enzymes activities i.e. PAL, POX, β -1, 3-glucanase activities, total protein and phenol contents. While control or distilled water treated seeds showed only 10–40% survival when grown in fungal infested soil (Mahapatra et al., 2015). Fungal forced inoculation study showed that *S. rolfii* derived elicitor and fungicide treated groundnut seeds showed higher germination rate, more vigor and less mortality rate in *S. rolfii*, *A. niger*, *A. flavus* and *A. parasiticus* infested soils (Amin, 2017).

11.4.7 Field experiments

Fungal elicitors also play significant role in inducing plant's growth and development along with disease resistance which leads to increased yield. The field experiments were carried out by several researchers to evaluate the efficacy of fungal culture filtrates on field grown plants for induction of defense mechanism.

The foliar application of *A. flavus* and *A. parasiticus* derived elicitors either treated once or twice given at vegetative or reproductive stage in field grown maize cultivar induced defense related enzymes i.e. PAL, POX, PPO, β -1,3 glucanase, catalase, chitinase, NR, NiR, glutathione reductase along with total protein, phenol, carbohydrate, chlorophyll and lignin content and lignin deposition in leaf tissue of elicitor treated plants compared to control plants. Salicylic acid accumulation was also increased in all the elicitor treated plants. Elicitor treated maize plants showed highest number of seeds/cob and cob weight than other treatments and control plants. Field grown elicitor treated plants showed the variation in the level of induction of various defense enzymes in different seasons but the enzyme activities remained high

throughout the experiment compared to control plants. SDS-PAGE analysis and Thin layer chromatography revealed various induced protein (chitinase and β -1,3 glucanase) and phenols (vanilic acid, ferulic acid, salicylic acid, cinnamic acid, gallic acid and coumaric acid) in fungal elicitor treated maize plants than control (Mahapatra et al., 2015). The effect of foliar application of *S. rolfii* derived elicitor at flower initiation stage and pegging and maturation stage on field grown groundnut plants was studied by Amin (2017) during 2011–14 and observed that the induction of various enzymes activities varied among the treatments at different time interval. After single or double foliar treatments with fungal elicitor, activity of various defense enzymes, antioxidant enzymes, protein, phenol, carbohydrates, chlorophyll and lignin content remained high throughout the experiment in treated plants compared to control plants. These foliar treatments showed induction of caffeic acid, catechol along with common phenols i.e. shikimic acid, gallic acid, vanilic acid, salicylic acid, ferulic acid and cinnamic acid in treated plants. SDS PAGE analysis also revealed the induction of additional proteins probably belonging to β -1,3 glucanase, PR-1 family having antifungal proteins and unknown PR proteins in elicitor treated plants. Induction of additional phenol and proteins in elicitors treated plants depends on the type of elicitor. Histological studies of elicitor treated leaf showed lignified epidermal cells and presence of more chloroplasts in palisade cells, more number of xylem elements in vascular bundle with lignified walls in comparison with control plant. There was not any disease incidence of collar rot or southern blight in elicitor treated plants compared to control at the time of harvest indicating broad spectrum of resistance against *S. rolfii* and *A. niger*. Various yield attributing factors were detected higher in elicitor treated plants i.e. 17–38% increase in yield in SRFCF and fungicide treated plants as compared to control plants. The results of physiology and biochemical parameter attained in various field study varied with different kharif seasons depending on the climatic condition and agronomic practices during 2011–2014. The higher content of oil was extracted from seeds harvested from SRFCF (44%) and fungicide (30%) treated plants compared to control (21%) plants. Nandini (2011) found that treatment with *S. rolfii*, *A. parasiticus* and *A. flavus* derived FCF triggered activity of PAL, POX, β -1,3-glucanase and increased content of total protein, phenol, lignin and salicylic acid in groundnut plants compared to untreated plants. Nandini (2011) observed that in field experiments carried out during 2006–2009, groundnut plants treated with *S. rolfii*, *A. flavus*, *A. parasiticus* derived FCF showed lowest disease incidence with healthy green appearance than control plants as well as seeds harvested from elicitor treated plants contained low aflatoxin in all varieties than control one. Aflatoxin content in stored groundnut seeds harvested from elicitor treated plants was also lower than control one with reduced disease incidence. Thus, these elicitor treatments were effective in disease control as well as increased yield and quality. The study evidently proved that pathogen (*S. rolfii* and *A. flavus*) derived FCFs induced resistance in different varieties of groundnut plants against further pathogen attack compared to MCW prepared from pathogen on the basis of induction of defense enzymes (Nandini et al., 2010; Nandini and Bariya, 2014).

11.5 Possible biochemical pathway of elicitation upon fungal elicitors application in plants

Based on the above observations, the following biochemical pathway can be assumed to be induced upon fungal elicitor treatment (Fig. 11.1). After the foliar application of various fungal components acting as elicitors, the hypersensitive response is triggered by the plants. During the plant pathogen interaction, the components of pathogen i.e. 'avr' gene product of pathogen binds with the receptor molecule i.e. 'R' gene product of host plant. Putative binding of these two components leads to the activation of cascade of signal transduction resulting in the variety of plant defense response. Within short time period, receptor activation causes an influx of calcium (Ca^{2+}) and hydrogen (H^+) ions into the cell while efflux of potassium (K^+) and chloride (Cl^-) ions (Nurnberger and Sheel, 2001). These increased cytosolic calcium (Ca^{2+}) concentration initiates the nitric oxide (NO) synthesis through pathogen induced nitric oxide synthase (iNOS) (Chandok et al., 2003). The plant pathogen interaction leads to the rapid production of reactive oxygen species (ROS) which in turn activates the hypersensitive response (HR) causing death of the cell surrounding the infection site thereby preventing the spread of pathogen. These ROS being the strongest oxidants lead to the lipid peroxidation and enzyme inactivation (Lamb and Dixon, 1997) resulting into the programmed cell death (PCD) at the site of infection. The above studies presented that the application of fungal derived elicitors showed HR response as necrotic lesions when applied on leaf surface. The induced activity of Nitrate reductase (NR) and Nitrite reductase (NiR) leads to the production of nitrogen containing primary and secondary products essential for growth and development of plants. Nitric oxide (NO) is assumed to be produced by an alternative pathway of NADPH dependent reduction of nitrite to NO catalysed by NR (Yamasaki et al., 1999). Simontacchi et al. (2013) has reviewed the role of nitric oxide (NO) in signaling pathways involved in plant growth, stomatal movement and in senescence. Nitric oxide plays a major role in

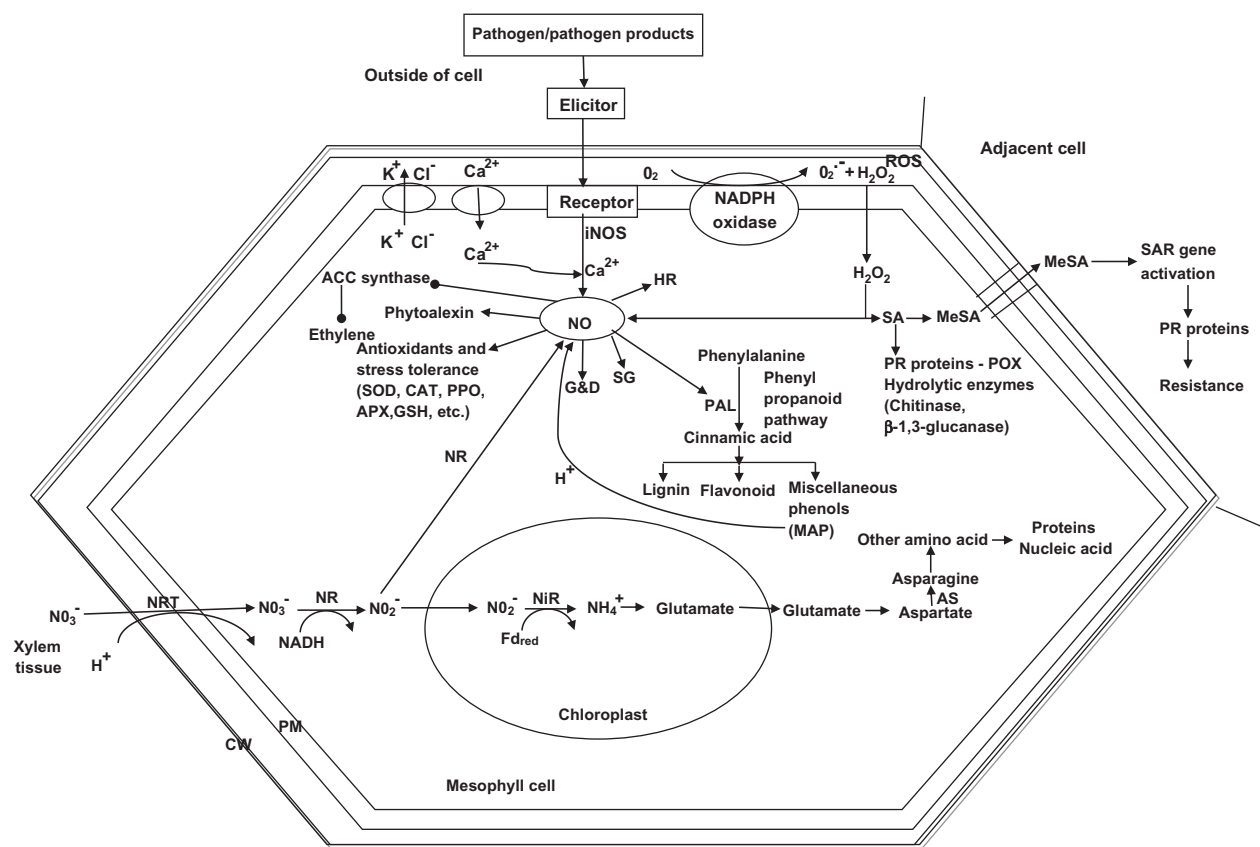


FIGURE 11.1 Possible biochemical reactions upon application of fungal elicitors in plants.

Abbreviations: iNOS-pathogen inducible nitric oxide synthase, NO-nitric oxide, NR-nitrate reductase, NiR-nitrite reductase, NO₃-nitrate, NO₂- nitrite, NRT- nitrate-proton symporters, HR- hypersensitive response, PAL- phenyl alanine ammonia lyase, SOD- superoxide dismutase, POX- peroxidase, APX- ascorbate peroxidase, PPO-polyphenol oxidase, CAT-catalase, SA-salicylic acid, MeSA- methyl salicylate, ROS-reactive oxygen species, PR-pathogenesis protein, ACC- 1-Amino cyclopropane carboxylate, MAP- malonic acid pathway, CW- cell wall, PM- plasma membrane, G&D- growth and development, SG- seed germination.

the early events of resistant response along with hydrogen peroxide (H_2O_2) for salicylic acid (SA) synthesis. SA in turn enables local resistance through the activation of PR-proteins ultimately leading to the systemic acquired resistance (SAR). Methyl salicylate (MeSA), methyl ester of SA transmits the signal of SAR to distant uninfected parts of the plant and even to the neighboring plants (Shulaev et al., 1997). The accumulation of SA probably leads to the production of PR-proteins i.e. chitinase and β -1,3-glucanase etc. Nitric oxide (NO) is also reported to induce activation of phenylalanine ammonia lyase (PAL) gene and PR-1 gene expression (Durner et al., 1998) which may activate the phenylpropanoid pathway leading to the production of various secondary metabolites such as phenolics, phytoalexins, lignin and flavanoids. Elicitor treated plants are reported to synthesize more phenols as compared to control plants along with lignified cell walls in mesophyll cells of leaves indicating development of resistance against pathogens. Nitric oxide (NO) seems to be a bi-functional modulator that is capable of either stimulating or inhibiting synthesis of ROS (Misra et al., 2010), Phytoalexin production (Leshem, 1996), breaking of seed dormancy and enhancing seed germination (Bethke et al., 2004), shoot and root growth (Zhang et al., 2003) and also blocks ACC synthase thereby preventing ethylene production. SAR is established within few days of elicitor treatment in plants via NO-ROS-SA-PR proteins imparting resistance to plants.

11.6 Conclusion and future directions

Focusing on the systemic resistance, fungal components can be considered as the best elicitors to control diseases of plants. Fungal elicitor treatments induce various defense related enzymes, PR proteins and phenols providing ample immunity to the plants to fight against the pathogens along with better growth, development and higher yield. Seeds or fruits obtained from the plants after fungal elicitor treatment meets more than the required quality parameters for the

export of the commodity. The research can help to understand the role and networking of signal molecules that generate systemic defense response in plants. Significant development of biotic elicitors that elicit the SAR in plants with enhanced disease resistance are the important outcome of these studies. This approach is an important compliment to classical breeding techniques for the screening of those resistance breeding lines.

Following advantages of elicitor treatments to induce systemic resistance in plants have been reported: reduced damage from various diseases, reduced environmental threats as elicitors target directly the crop plant and not the other organisms so risk of development of resistant pathogen is lower than that of fungicides, elicitors treatment can be given as protective agrochemicals with the current spraying technology, cost-effective, more durable in its effect by not requiring repetitive treatments, reduced the use of chemical fungicides and could be a better alternative to genetically modified (GM) plants as it possess lower ecological risks than GM plants. Thus, fungal components can be used as elicitors to induce plant defense mechanism against a broad spectrum of pathogens. Further research may be focussed to find out the structural elucidation of the active compound present in the fungal component and its synthetic production for commercial use as an elicitor. However, scale-up studies are needed for commercial production as well as detection of genes responsible for the induction of resistance in plants after application of elicitor.

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Chapter 12

Versatility of *Trichoderma* in plant disease management

Laith Khalil Tawfeeq Al-Ani^{1,2} and Aeshah Mhana Mohammed³

¹Department of plant protection, College of Agriculture, University of Baghdad, Baghdad, Iraq, ²School of Biology Science, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia, ³Department of Chemistry, College of Education for pure science (Ibn al-Haytham), University of Baghdad, Baghdad, Iraq

Chapter outline

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12.1 Introduction

Trichoderma is the most interested genus in the fungi group and biological control agents. This genus is more useful for plant and also for the ecosystem. *Trichoderma* have many mechanisms that can utilize in enhancing the typical growth and best production. The mechanisms are including mycoparasitism, competition, enhancing the plant growth, producing antibiotics, and able to induce the resistance and defence of plant. The huge ability of *Trichoderma* are utilized to enhance the plant health, and agriculture production.

However, *Trichoderma* is a better helping microbe to save the plant from the influence the plant pathogens. Many studies showed the high ability *Trichoderma* in control of the plant pathogens. Therefore, *Trichoderma* considers an alternative method the chemical pesticides. The alternative methods were showing high efficacy in the control the plant pathogens (Al-Ani 2006; Al-Ani and Salleh 2010; Mohammed et al., 2011, 2012, 2013, 2014; Al-Ani and Al-Ani 2011; Al-Ani et al., 2012, 2013, 2018; Al-Ani 2017a,b; Al-Ani and Albaayit, 2018a,b; Al-Ani 2018a,b; Al-Ani 2019a,b,c,d,e,f). Two isolates of *T. harzianum* were showing high antagonistic against *Fusarium* pathogen of tomato (El-Katatny et al., 2011). Many isolates of *Trichoderma* sp. were used against *Ganoderma* disease (Naher et al., 2012; Musa et al., 2017). *T. harzianum* used to control the soil borne diseases (Yousef et al., 2018). *T. gamsii* was useful in controlling of root rot disease (Chen et al., 2016). *T. harzianum* and *T. koningii* were antagonistic three soil borne diseases such as *Pyricularia oryzae*, *Ganoderma boninense*, and *Fusarium oxysporum* (Naher et al., 2017).

Interestingly, *Trichoderma* can utilize to control the plant pests potentially. Begum et al. (2018) used *T. harzianum* against Aphids. *T. hamatum* was useful to control the cotton aphids (Mona et al., 2016). The conidia of *T. harzianum* are found effective on tropical bed bugs (Ab Majid et al., 2015; Zahran et al., 2017). *Trichoderma* sp. showed the efficacy in controlling the cocoa pod borer (Amin et al., 2014). *T. atroviride* caused the mortality for the larvae of insect *Drosophila melanogaster* (Atriztán-Hernández et al. 2018). *Trichoderma* sp. led to cause the mortality for the larvae of beetle (Nasution et al., 2018). *T. longibrachiatum* was succeeded to control of *Leucinodes orbonalis* in the field (Ghosh and Pal 2016). They found most fungi isolated from the dead larvae of cowpea pod borer were *Trichoderma* (Omoloye et al., 2015). In additional, there is another role of *Trichoderma* using in controlling the weeds (Al-Ani 2019b).

Surprisingly, three *Trichoderma* isolates showed the ability in controlling two weeds of wheat (Javaid and Ali 2011). The treatment with *T. koningiopsis* led to reduced plant growth in *Euphorbia heterophylla* plants (Bordin et al., 2018). *T. pseudokoningii*, *T. reesei*, *T. harzianum*, and *T. viride* were showing the efficacy in reducing the seed germination and growth of root and shoot of parthenium weed (Javaid et al., 2013). *T. virens* was inhibited the seed germination of two weeds such as *Setaria viridis* and *Amaranthus retroflexus* (Hutchinson 1999). *T. virens* was able to reduce growth of two weeds such as redroot pigweed and broadleaf weeds in the fields (Héraux et al., 2005). However, *Trichoderma* can be useful for the plant through enhancing the plant growth and improving the soil characterizes. Windham et al. (1986) found the ability of *Trichoderma* enhancing the germination of seeds and increase in dry weight of shoot and root for the tomato. *Trichoderma* showed the efficacy in improvement of plant height, photosynthesis, and grain yields of rice (Doni et al., 2017). Four isolates of *Trichoderma* enhanced the plant vigor of banana (Al-Ani 2017b). *T. longibrachiatum* T6 strain enhanced the plant growth and tolerance of wheat plant against the salt stress (Zhang et al., 2016b). Therefore, *Trichoderma* can utilize to control of plant pathogen, insects, and weeds, as well as, enhancing the plant growth. This may be providing a chance to use *Trichoderma* as instead of chemical synthetic pesticides and fertilizer. This considered the important role of *Trichoderma* according to utilize it in control of plant pathogens and pests in the soil and infect different parts of the plant, as well as, improving the plant growth.

12.2 Soil borne diseases

Soil-borne diseases are huge problem for agriculture production. The soil is containing many harmful genres of plant pathogens such as nematodes, bacteria, and fungi. In same time, the soil is a better medium for *Trichoderma* to grow and conducts hostile activities against the plant pathogens. The seed treatment with *T. harzianum* are one the best method to provide a protection for the plant infection with soil borne pathogens such as *Rhizoctonia solani* caused root rot and damping off in the field (Ruppel et al., 1983). *T. viride* produced several of fungal cell wall-degrading enzymes, and suppression of the disease symptoms (Grosch et al., 2007). *T. viride* showed several mechanisms against soil-borne pathogen *R. solani* including; (1) inhibiting the melanin formation for the fungal cell wall and the sclerotia germination, (2) mycoparasitism, and (3) antibiosis (Grosch et al., 2007). However, three factors such as substrate of media, pH affected the ability of *T. harzianum* to inhibit growth of soil-borne pathogens including *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, and *Fusarium oxysporum* (Meena and Meena 2016).

12.3 Seed diseases

Many plant pathogens are causing the diseases for the plant seeds. Some plant pathogens such the fungal pathogen secrete several harmful secondary metabolites such as mycotoxins by infection the seeds (Bennett and Klich 2003; Attitalla et al., 2010a,b). The role of *Trichoderma* become by covering the seeds and colonizing the surface of the seeds and then protect for seeds. The special formulation of a spore suspension is providing good protection against infection with plant pathogens. The mechanisms of *Trichoderma* in controlling seed diseases comprise, (A) the inhibition for the growth of plant pathogens by secreting the enzymes and secondary compounds, (B) Inducing the seeds to produce antibiotics against seed-borne plant pathogen, (C) Competing with plant pathogens by colonizing the infection sites and on the nutrients such as carbon and nitrogen. *Trichoderma* is having the ability of production a siderophore as the tool for competing on iron that reducing the growth and pathogenicity. Al-Ani et al. (2019a,b,c,d,e,f) detected the ability of four *Trichoderma* isolates producing a siderophore and affecting on plant fungal pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc*TR4). and (D) Stimulating the germination quickly that provide a chance to escape from the plant pathogens.

Indeed, they made two formulations including a slurry formulation and starch solutions of two species *Trichoderma* such as *T. harzianum* and *T. koningii* (Adekunle et al., 2001). They found these formulations reduced the disease of damping off that caused by plant fungal pathogens *Macrophomina phaseolina* (Adekunle et al., 2001). Coşkuntuna and Özer (2008) mention to possible enhancing the protection of onion seeds against *F. oxysporum* f. sp. *cepae* by coating with conidia *T. harzianum* KUEN 1585 strain and treated with extract. The antifungal compounds found in the treated seeds with *Trichoderma* through extracting of the treated seeds with 95% ethanol solvent (Coşkuntuna and Özer 2008). The seeds of cane palm *Chrysalidocarpus lutescens* soaked with a conidia suspension of two *Trichoderma* species including *T. harzianum* and *T. viride* showed high antagonism against *Helminthosporium* sp. and enhanced the seed germination (Jegathambigai et al., 2009). The seeds sunflower treating with talc formulation of *Trichoderma* was shown high resistance against the *Plasmopara halstedii* that caused downy mildew disease (Nagaraju et al., 2012). *T. harzianum* showed high antagonistic against *Phytophthora capsici* through treating the pepper seeds (Sofian et al., 2013).

Yousef et al. (2018) found the ability of faba bean seed to confront the plant fungal pathogens *Rhizoctonia solani* increased after treating with conidia suspension of *T. harzianum*. They detected many antifungal in crude extract of conidia suspension such as (I) 2,3-butanediol, (II) phenylethyl alcohol, (III) Decane, 2,4,6-trimethyl-, (IV) acetic acid, and (V) 1 H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-,cis- (Yousef et al., 2018).

12.4 Root diseases

The role of *Trichoderma* in reducing of root disease and affecting on plant pathogens is using similar mechanisms like as that used in controlling of seed diseases. The root treatment is providing a better chance to control of plant pathogens. *Trichoderma* is having the capability to compete on infection sites and for nutrients with pathogens on the root surface. As well, other mechanisms such as parasitism, secrete secondary compounds, enzyme, induce the defence and resistance can be useful against many plant pathogens. *Trichoderma atroviride* strain SJ3-4 is a transgenic strain showing high effect on two important pathogens *Pythium* and *Rhizoctonia* (Brunner et al., 2005). *T. gamsii* YIM PH30019 affected several plant pathogens causing the root-rot disease for the medicinal herb *Panax notoginseng* (Chen et al., 2016). The ability of *Trichoderma* produce cell wall degrading enzymes (CWDEs) useful in the inhibition of plant pathogens through contact. Saravanakumar et al. (2016a) detected four CWDEs such as chitinase, protease, β (1-3) glucanase and cellulase in the crude extract of *T. asperellum*. As well, the elucidating of secondary compounds is appearing through affecting the pathogenicity of the plant pathogens. A secondary compound 1,6-disphenylhexane-1,3,4,6-tetrone of *T. asperellum* showing efficacy in effect on the protein both of growth and pathogenicity for *Fusarium oxysporum* f. sp. *cucumerinum* (Saravanakumar et al., 2016b). A special substrate found in solid state fermentation such as maize grit enhanced the production of *T. asperellum* strain RSTV04 (Babul Reddy et al., 2017). This strain RSTV04 inhibited the mycelium growth of *Fusarium oxysporum* very efficacy (Babul Reddy et al., 2017). *T. harzianum* showed high inhibition for two soil-borne pathogens *Pythium ultimum* and *Phytophthora capsici* and reduced the size of lesion on the tomato root (Uddin et al., 2018). Surprisingly, it can detect the metabolism change in the host leaf through interaction between *Trichoderma* and the plant root pathogen. Mayo-Prieto et al. (2019) found many significant changes in the bean leaf after treating the bean root with antagonistic fungi as *Trichoderma velutinum* and the root pathogen *Rhizoctonia solani*. Several metabolisms such as flavonoids, terpenes, carbohydrates, phenols, amino acids, and peptides have been detected using the LC-MS analysis that suggestion was a plant defense response (Mayo-Prieto et al., 2019).

12.5 Stem diseases

The role of *Trichoderma* in the reduction the stem diseases has been detected. The production of the secondary metabolites and enzymes, and also induce the defense and resistance in the plant may be more effective in confronting the plant pathogens causing the stem diseases. The best method to use *Trichoderma* in controlling of stem diseases is by treating the plant root with a special formulation of conidia suspension and also culture extracts for *Trichoderma*. However, Zhang et al. (2016a) detected the potential control the plant disease *Sclerotinia* stem rot for soybean that causing by plant pathogen *Sclerotinia sclerotiorum*.

Indeed, four methods were determined to control *Sclerotinia* stem rot; (I) Mycoparasitism, (II) Secreting the antifungal, and (III) Induce systemic resistance (Zhang et al., 2016a). They found four isolates such as *T. harzianum*, *T. brevicompactum*, *T. virens*, and *T. asperellum* controlling the plant pathogen *Ganoderma boninense* causing basal stem rot for oil palm (Musa et al., 2017). Musa et al. (2017) mention for some effective mechanisms of *Trichoderma* against the stem pathogen were comprising mycoparasitism, production of siderophores and hydrolytic enzymes such as chitinase, cellulase, β -1, 3-glucanases, and protease. The ability of *T. harzianum* P-11 found in inhibiting the growth plant pathogen *Sclerotinia sclerotiorum* that caused white mold of common bean (*Phaseolus vulgaris*) (Bedine Boat et al., 2018). However, the dipping root and soil application with a special formulation for *T. asperellum* reduced the stem rot disease in carnation that caused by *Sclerotinia sclerotiorum* (Vinodkumar et al., 2017).

12.6 Leaf diseases

Trichoderma has a clearance role in controlling foliar pathogens such as *Alternaria*, *Botrytis*, *Colletotrichum*, and others genera. The leaf of the plant can spray with conidia suspension or culture filter, or together of *Trichoderma* possible induced the ability of a plant to control of plant pathogens. The conidia suspension of *T. harzianum* strain 1295-22 could be applied by spray applications and control the plant pathogen *Pythium graminicola* (PRR-8) causing brown

patch, root rot, and dollar spot for creeping bentgrass (Lo et al., 1997). The metabolites of *Trichoderma* are more effective on the foliar pathogens. Brunner et al. (2005) usage *T. atroviride* strain SJ3-4 (a homologous chitinase (nag1) promoter) showed very efficient in inhibiting of spores germination and induce systemic resistance against the foliar pathogen *Botrytis cinerea*. *T. harzianum* induced the defense in the cabbage leaf by increasing the transcription levels of enzymes such as chitinase and ascorbate peroxidase (Liu et al., 2016). The metabolites of *T. virens* and *T. atroviride/petersenii* induced the defense-related genes and made the change in the plant hormone that causing the inhibition for the foliar pathogen *Phytophthora* spp. (Bae et al., 2016).

12.7 Fruit diseases

Trichoderma is producing several antibiotics, enzymes, and secondary metabolites that utilizing to control of fruit pathogens. *T. asperellum* T8a produced cellulases a useful enzyme against fruit pathogen *Colletotrichum gloeosporioides* ATCC MYA 456 of mango (de los Santos-Villalobos et al., 2013). Mycoparasitism and induce the defense and resistance in plant are useful to control fruit pathogens. Many proteins of *T. harzianum* utilized in mycoparasitism on the mycelium and induce the plant defense against fruit pathogen *Guignardia citricarpa* that caused citrus black spot (de Lima et al., 2017). *T. gamsii*, *T. atroviride*, and *T. hamatum* affected the plant pathogen *Fusarium oxysporum* f. sp. *cepae* in the field which caused fusarium basal rot for onion (Bunbury-Blanchette and Walker 2019).

12.8 Postharvest diseases

Many plant pathogens cause rot, spot, and anthracnose for the agricultural products after harvest. The isolated of *T. harzianum* (TrH 40) was showed high efficacy against three plant pathogens *Colletotrichum gloeosporioides*, *Botryodiplodia theobromae*, and *Gliocephalotrichum microchlamydosporum* for rambutan by mycoparasitism (Sivakumar et al., 2000). The mango fruits treated with conidia suspension of *T. viride* inhibiting growth of several the post-harvest pathogens of mango such as *Pestalotia* sp., *Colletotrichum gloeosporioides*, *Aspergillus flavus*, *Lasiodiplodia theobromae*, *Rhizopus stolonifer*, *Macrophomina phaseolina*, and *A. niger* (Bhuvaneswari and Rao 2001). *T. viride* showed the high ability in competition and antagonistic against postharvest pathogens of cassava roots rot such as *Rhizopus oryzae*, *Aspergillus flavus*, *Fusarium solani*, and *Botryodiplodia theobromae* (Ubalua and Oti 2007). *T. viride*, *T. koningii*, and *T. harzianum* were antagonistic against postharvest pathogen *Colletotrichum musae* and *Lasiodiplodia theobromae* for the banana very efficacy both of cold and in root temperature storage (Sangeetha et al., 2009).

Indeed, two isolates of *T. harzianum* showed the capability of inhibition for different post harvest pathogens of tomato by producing the volatile and non volatile, and mycoparasitism (El-Katatny et al., 2011). *T. longibrachiatum* and *T. viride* showed a high efficacy in mycoparasitism the postharvest pathogen *Colletotrichum gloeosporioides* through treating the papaya fruit 24 hours with conidia of two isolates of *Trichoderma* before the pathogen (Landerio Valenzuela et al., 2015). Four isolates of *Trichoderma* included *T. longibrachiatum* (4088), *T. virens* (255C1), *T. asperellum* (SF04), and *T. harzianum* (THP) very efficient in inhibiting the growth of postharvest pathogen *Phytophthora palmivora* for the papaya fruit through applying in the different ways (Oliveira et al., 2018). In addition, the culture filter of many *Trichoderma* spp. such as *T. hamatum*, *T. polysporum*, *T. pseudokoningii*, *T. harzianum*, and *T. longibrachiatum* are useful in controlling of postharvest pathogen *A. niger* (Terna et al., 2016). The dipping apple fruit in formulation of *T. harzianum* for 30 s protected the apple from postharvest pathogen *Penicillium expansum* for the up of 2 months (Batta 2004).

12.9 Entomopathogenic fungi

The different mechanism of *Trichoderma* such as parasitism and produces the secondary metabolites and enzymes affects on the insect and can put within an entomopathogenic fungi group. *Trichoderma* can attack and causes mortality for eggs, larvae, pupa, and adults of insect. For the eggs, *T. harzianum* caused the mortality to pest *Bemisia tabaci* by mycoparasitism (Krauss et al., 2004). *Trichoderma* spp. caused an egg mortality of cocoa pod borer *Conopomorpha cramerella* between 84% and 89% in two type media for powder and liquid (Amin et al., 2014). For the larvae, *Trichoderma* spp. caused high mortality at 96.67% for larvae of horn beetle *Oryctes rhinoceros* on oil palm (Nasution et al., 2018). *Trichoderma* spp. was an entomopathogen fungus for the pest larvae of cowpea pod borer, *Maruca vitrata* (Omoloye et al., 2015). The first detected *T. longibrachiatum* as entomopathogen fungus for the larvae insect of *Leucinodes orbonalis* (Ghosh and Pal 2016). *T. atroviride* caused mortality for the fungivory larvae of *Drosophila*

melanogaster (Atriztán-Hernández et al., 2019). For the pupa, *T. harzianum* secreted some secondary metabolisms and enzymes causing the toxicity for pupa and larvae of pest *Aedes aegypti* L. (Sundaravadivelan and Padmanabhan 2014).

Indeed, for the adults, *T. harzianum* was a pathogen for a tropical bed bug *Climex hemipterus* (Ab Majid et al., 2015; Zahran et al., 2017). The extract of culture filter of *T. harzianum* affected the insect pest aphids *Macrosiphum rosae* (Begum et al., 2018). However, *Trichoderma* can affect the physiological activity of insect. *T. hamatum*, an entomopathogen for *Aphis gossypii* (the cotton aphid) and caused different quantitative changes in the total soluble protein such as the carbohydrates hydrolyzing enzymes and transaminase enzymes (Mona et al., 2016).

12.10 Nematophagous fungi

Trichoderma can parasitic and produce a secondary metabolism and enzymes that cause the death for the nematode that calls nematophagous fungi. *T. asperellum* deactivated the densities of second stage juvenile and *T. brevicompactum* affected on the eggs production of root-knot nematodes *Meloidogyne incognita* in the root (Affokpon et al., 2011). *Trichoderma* such as *T. harzianum* and *T. viride* affected on the reproduction and root galling of the nematode *Meloidogyne javanica* in the tomato host (Al-Hazmi and TariqJaveed 2015). *T. longibrachiatum* showed the ability to parasitism on cysts for nematode *Heterodera avenae*. *T. longibrachiatum* produced the high numbers of conidia that are growing on the surface of the cyst and secreted the extracellular chitinase enzymes (Zhang et al., 2014). *T. harzianum* could colonize the second-stage juveniles with affecting on cysts recovered from soil and reduction in reproduction of the potato cyst nematode *Globodera pallida* (Contina et al., 2017). *T. harzianum* attacked the eggs and juveniles and induced the plant defence against root-knot nematode *Meloidogyne incognita* (Singh et al., 2017).

However, Yang et al. (2012) detected three secondary metabolisms useful as nematicidal activity including; (A) 1 β -vinylcyclopentane-1 α ,3 α -diol, (B) 6-pentyl-2H-pyran-2-one and (C) 4-(2-hydroxyethyl)phenol against three plant-parasitic nematodes *Caenorhabditis elegans*, *Panagrellus redivivus*, and *Bursaphelenchus xylophilus*. In addition, *T. harzianum* showed an expression for two chitinolytic enzymes chi18-5 (chit42) and chi18-12 (chit33) through the parasitism activity on the eggs of plant-parasitic nematode *Caenorhabditis elegans* (Szabó et al., 2012).

12.11 Mycoherbicidal

Trichoderma spp. are having some aggressive strains for the weeds that can use as mycoherbicide. Muthumeenakshi et al. (1998) mention to 15 *T. harzianum* very aggressive strains on the weeds. *Trichoderma* spp. can inhibit the growth and emergence weed by treating the seed and soil. *T. virens* produced the antibiotic compounds such as viridiol that was a phytotoxin affecting on the seed germination and growth of two weeds including *Setaria viridis* and *Amaranthus retroflexus* through mixing *Trichoderma*, first test, with the seeds and another test with the soil fields (Hutchinson 1999). They extracted the culture filter (Malt extract broth) of *T. pseudokoningii*, *T. reesei*, *T. harzianum*, and *T. viride* showing high inhibition for seed and seedling of *Parthenium hysterophorus* L. (Parthenium weed) (Javaid et al., 2013). The culture filter of *Trichoderma* can be used for weeds. The culture filter (CF) of four *Trichoderma* species such as *T. harzianum*, *T. pseudokoningii*, *T. reesei*, and *T. viride* was extracted by using the solvent n-hexane and ethyl acetate. CF sprayed on the leaf of wheat weeds *Rumex dentatus* L. that caused high toxicity for this weed (Javaid and Ali 2011). The mycelium extract of *T. harzianum* showed the ability to cause the toxicity for *Lemna minor* (Duck weed) (Begum et al., 2018). The extract of *T. koningiopsis* was contained several effect enzymes including amylases, peroxidases, lipases and cellulases that phytotoxicity for the weed *Euphorbia heterophylla* (Bordin et al., 2018).

12.12 Biofertilizer

Trichoderma play a role in stimulating the plant growth and provide the useful elements for the plant to be a biofertilizer. *Trichoderma* spp. secreted a growing -regulating that increased the seed germination and dry weight for shoots and stems of tomato (Windham et al., 1986). Four isolates of *Trichoderma* spp. enhanced the plant vigor of the banana tree (Al-Ani 2017b). The shoot numbers, flower yield, and stalk length enhanced after treating the root and soil surrounding of a carnation with the talc formulation of *T. asperellum* (Vinodkumar et al., 2017). *T. asperellum* showed the increased in; (A) plant vigor, (B) physiological functions such as stomatal conductance, photosynthetic rate, and chlorophyll content, and (C) the yield production (Doni et al., 2017). As well, Doni et al. (2017) detected the reduction in concentration of internal CO₂ and rate of transpiration that leading to the efficient in converting CO₂ into the mass of carbohydrates. In addition, Akladious and Abbas (2014) detected the ability of *T. harzianum* to improve the plant vigor such as; (A) growth, (B) the content of chlorophyll, nucleic acids, phytohormone, starch, and total protein for the maize.

T. harzianum enhanced the plant vigor of tomato that useful to reduce the effect of two plant pathogens including *Pythium ultimum* and *Phytophthora capsici* (Uddin et al., 2018).

Surprisingly, *T. longibrachiatum* induced the increase in gene expression and the antioxidant peroxidase, enzymes-superoxide dismutase, and catalase that were enhance the plant growth and providing a protection for wheat seedling against salt stress (Zhang et al., 2016b). *T. harzianum* increased the nutrient content and yield production of tomato (Nzanza et al., 2012). The culture filter of *Trichoderma* is contenting many useful enzymes and secondary metabolites that improve the plant vigor of the host plant. Zhang et al. (2016a) found the culture filter of *T. harzianum* exhibited an interesting role in increased the activities of superoxide dismutase, catalase, and peroxidase, the hydrogen peroxide (H_2O_2) with contenting of the superoxide radical and chlorophyll in the soybean plant. The secondary metabolite of *T. harzianum* included several useful compounds like as chrysophanol compound that induced the photosynthesis and plant growth of cabbages (Liu et al., 2016). Siderophores, Indole-3-acetic acid, and gibberellins, are producing by some species of *Trichoderma* that useful for enhance the plant growth (Bezuidenhout et al., 2012; Hermosa et al., 2012; Fu et al., 2015; Al-Ani 2017b; Mukherjee et al., 2018; Zhou et al., 2018; Al-Ani et al. 2019a). In addition, different volatile compounds produced by *Trichoderma* such as *T. pseudokoningii* was promoted the growth of the root lateral of the plant host (Lee et al., 2016).

12.13 Conclusion

The consideration for the *Trichoderma* genus is a best useful fungus compared with other fungi to show many mechanisms that enhance the plant health. The genome of *Trichoderma* is having the ability to confront the pathogens and pests of the plant, as well as, improvement the plant growth. The different mechanisms of *Trichoderma* are showing high efficient in controlling the diseases in different parts for the plants. *Trichoderma* is showing the capability in usage of the parasitism and non parasitism against plant pathogens in the soil, rhizosphere, and pathogens causing the root diseases. Indeed, the preparation of the special formulation of conidia suspension of *Trichoderma* showed the high efficient against many plant diseases by treating the soil, seed and root plant. The conidia germination are useful to parasitism with induced the defense and resistance of the plant against the plant pathogens. The culture filter of *Trichoderma* is effective for controlling plant diseases. The important factors are playing against plant pathogens including the growth inhibition, and induce the defense and resistance in the host plant.

On the other hand, the conidia suspension and culture filter of *Trichoderma* are showing the efficacy in the change of the physiology and causing the mortality for many insects and nematodes. For the insect, *Trichoderma* is able to the growth and colonizes on the surface of the egg, larvae, pupa, and adult insect. For the nematode, the eggs, cyst, second stage juvenile, and adult of nematode. The culture filter of *Trichoderma* is containing some enzymes such as chitinase, and proteinase that impact on the outer wall of eggs, and adult insects and nematodes. As well, many secondary compounds with enzymes in culture filter are causing the cytotoxicity for insects and nematodes. Interestingly, CF of *Trichoderma* considers to be the phytotoxicity factor for weeds. Therefore, this culture filter can extract with some solvent and use it as herbicidal for inhibiting the seed germination and the seedling emergence of weeds. While, other benefit can be *Trichoderma* useful to plants being by improving the plant vigor. *Trichoderma* affects the plant physiology and induces the plant to utilize the energy coming of photosynthesis to convert the carbon to carbohydrates. The increase in all parameters of growth for the plant by treating the plant with *Trichoderma* indicates to potential using this genus as alternative method of chemical fertilizer.

Finally, it is possible to prepare the conidia suspension of *Trichoderma* in optimum concentration that the best concentration almost between 1×10^4 to 1×10^6 cfu/mL. Then, conidia suspension of *Trichoderma* (TCS) can use in control of plant pathogens and pests as the best alternative method of chemical synthetic pesticides and fertilizer due to the soil and rhizosphere is an appropriate habitation for the *Trichoderma* genus to grow and do all activities. The adding of TCS to soil and rhizosphere is around the host plant and also for the fields before or after planting that provide a chance of protection against the plant pathogens, pests, and weeds, as well as, enhancement the plant vigor. While the culture filter (PDA or other media) of *Trichoderma* (TCF) can utilize by extracting with the special solvents such as ethanol, butanol, n-hexane, and ethyl acetate, then it utilizes as instead of synthetic chemical pesticides and fertilizer. The usage of TCF is differing from the conidia suspension. TCF is preparing with an appropriate concentration then adding to seeds, seedling, and leaf, which enhance the germination of seeds, growth of seedling, induce the defense and resistance of plant, and affecting on the plant pathogens, pests, and weeds. The usage of TCS or TCF is considering the best formula possible to utilize in the greenhouse and fields that saving a chance for the ecosystem from reducing any the residue of chemical synthetic pesticides and fertilizer.

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Chapter 13

Knock, knock-let the bacteria in: enzymatic potential of plant associated bacteria

Lorena Carro¹ and Esther Menéndez²

¹Microbiology and Genetic Department. University of Salamanca. Salamanca, Spain, ²ICAAM - Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Laboratório de Microbiologia do Solo, University of Évora, Évora, Portugal

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13.1 Introduction

Plants are not considered as whole individual independent entities any more, being now considered as whole communities at which microorganisms play fundamental roles; thus, plant microbiomes should be studied together with their host plants (Carro and Nouioui, 2017). The interactions between plants and their surrounding habitants are in constant readjustments and hundreds of microorganisms reach plant tissues every day. Since a few years, it has become clear that an optimum equilibrium in these processes is fundamental to maintain a correct health status of the plants, with small distortions generating the development of diseases or sub-developed conditions. The interaction of bacteria with plants have been focused for years on three main groups: (i) nitrogen fixing interactions of rhizobia with legumes and to a lesser extent of *Frankia* with actinorhizal plants, (ii) symbiosis with arbuscular mycorrhizae, and (iii) pathogenesis (Farrar et al., 2014). However, metagenomic studies opened a door to a whole diversity of interactions between microorganisms and plants, becoming the focus of study of many groups around the world (Dong et al., 2018; Navrátilová et al., 2018; Wang et al., 2018). In the same line, the study of enzymes, and specifically, hydrolytic enzymes have been mainly related to plant pathogens and their colonization methods, including the degradation of plant tissues at final steps (King et al., 2011; Yarullina et al., 2016). Nevertheless, this new idea of different microorganisms able to colonize plant tissues without side effects for the plant has originated the search of other functions for these compounds, exemplified by their role as recognition molecules. Mainly when their expression in colonization and endophytic life, contrary to what was previously expected for these enzymes, was more frequently found up-regulated than down-regulated (Zhang et al., 2015). Endophytes are able to live asymptotically inside plants at any stage of growth, providing additionally resources for the plants with the production of enzymes, nutrients and secondary metabolites that can be used by the plant to protect itself from biotic and abiotic stresses. Many of these enzymes produced by the bacteria are extracellular enzymes, generating their activities outside of their cells, with direct implications for their environments, or in the case of endophytes, for their host (Khan et al., 2017). Between the most commonly studied enzymes previously detected in beneficial bacteria are amylases, cellulases, chitinases, lipases, pectinases, proteases, chitinases, and xylanases (Fig. 13.1), which will be the focus of this study.

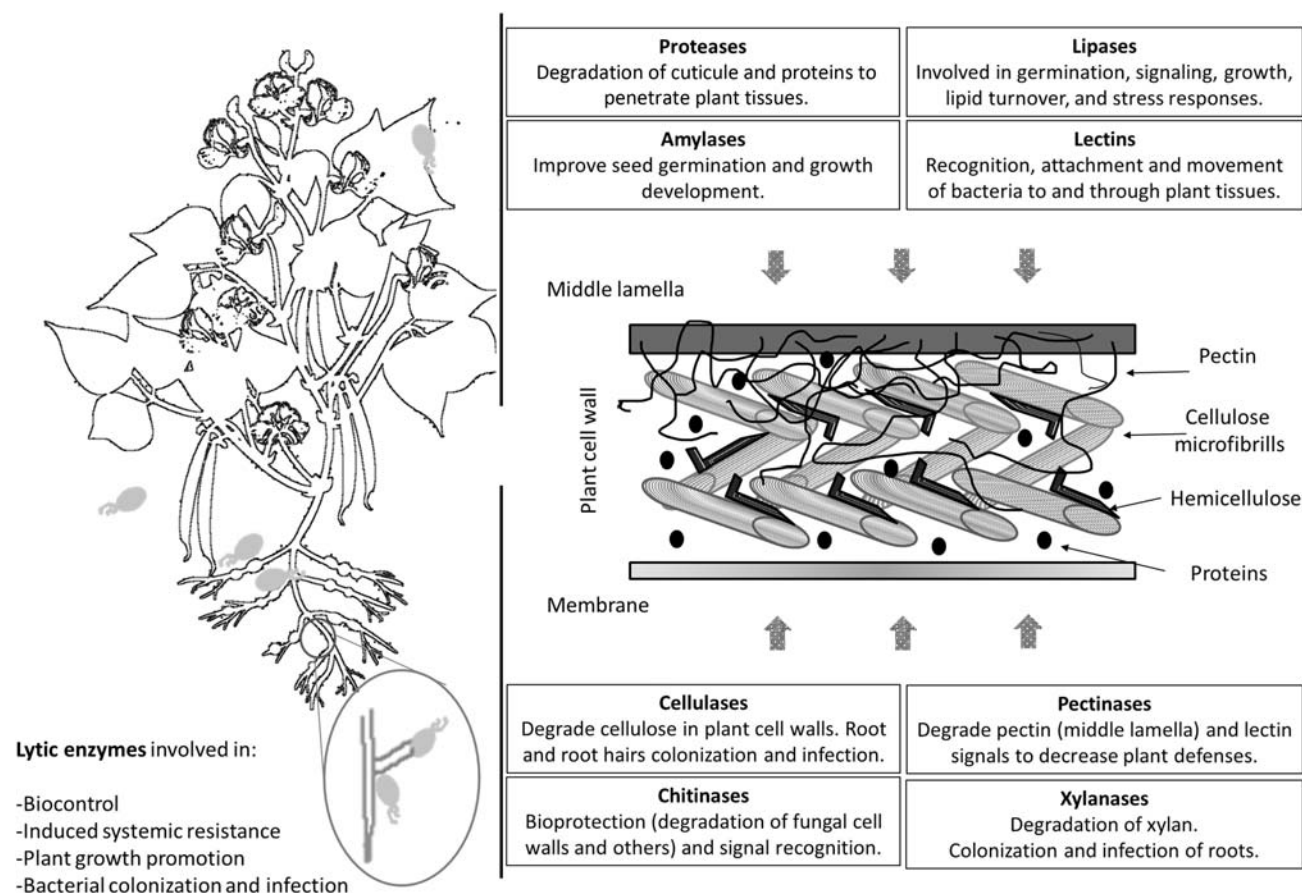


FIGURE 13.1 Main hydrolytic enzymes implicated in plant colonization and plant-bacteria interaction and their mode of action.

13.2 Amylases

Amylases are a class of enzymes that catalyse the hydrolysis of starch into glucose and maltose sugars. Three main groups of amylases have been described: α -amylases (EC 3.2.1.1), hydrolase enzymes that catalyse the hydrolysis of α -1, 4-glycosidic linkages producing glucose and maltose; β -amylases (EC 3.2.1.2), exo-hydrolases that hydrolyzed α -1, 4-glucan linkages into maltose units; and γ -amylases (EC 3.2.1.3), enzymes that cleaves $\alpha(1-6)$ and $\alpha(1-4)$ glycosidic linkages yielding glucose (Sundarram and Murthy, 2014). Amylases are constitutive enzymes which require calcium to stabilize its conformation and are tolerant to acidic conditions. However, its action could be inhibited by heavy metals (Yarullina et al., 2016). They could be found in several taxonomic groups, including fungi, oomycetes and bacteria. Within the bacteria amylases, some calcium-independent amylases have been described, although their tolerance to temperatures are more limited (Yarullina et al., 2016). Although the production of these enzymes have been mainly related to the penetration and degradation of plant tissues by pathogens, latter studies have confirmed its induction during germination of several plants by growth promoting bacteria (Islam et al., 2016). During the germination process, amylases are crucial for the hydrolysis of seed's endosperm, rich on starch, which allow the development providing the necessary energy for root and shoot development. Therefore, amylase induction and amylase production of plant growth promotion bacteria (PGPB) can improve plant establishment and general growth of the plants (Duarah et al., 2011). Moreover, the induction of α -amylases have been also related to the production of phytohormones, able to stimulate the plant growth, as for example the gibberellins (Kaneko et al., 2002).

It has been observed for several PGPB microorganisms belonging to distant phylogenetic taxa that amylases production is variable at different growth stages (Khan et al., 2017). Although it has been detected in many endophytes and its production is also over-expressed in contact with the plants, there are almost no studies showing its direct implication in the penetration of plant tissues. One of the hypotheses is that amylases help other enzymes in the formation of pores within roots, stems or leaves to allow bacterial colonization. However, more studies should be done to confirm at which level these enzymes are implicated in the colonization or just as helpers in tissues recycling.

13.3 Cellulases, pectinases and xylanases

Cellulase and xylan are the most abundant polysaccharides forming part of the plant cell walls. Several enzymes acting alone or in synergy are required to deconstruct those polysaccharides (Nguyen et al., 2018). The term cellulase is extensive to 3 different kinds of enzymes: endoglucanases (endo-1,4- β -D-glucanase; EC 3.2.1.4), which cleave β -1,4-glucan linkages, exoglucanase (exo-1,4- β -D-glucanase; EC 3.2.1.91/176), which degrade the rest of the polysaccharide chain originating cellobiose disaccharides and β -glucosidases (EC 3.2.1.21), which catalyse the breakdown of cellobiose disaccharides (Menéndez et al., 2015). These enzymes act in synergy in order to hydrolyze cellulose. There are also 3 types of xylanases: endo- β -1,4-xylanases (EC 3.2.1.8), which target the xylan backbone in plants and algae, endo- β -1,3-xylanases (EC 3.2.1.32), which act just in algae and finally, β -xylosidases (EC 3.2.1.37), which degrade xylobiose (Nguyen et al., 2018).

Pectin is a complex polysaccharide and one of the most abundant components of the middle lamella and primary cell walls (Fig. 13.1). Also, it is present in less quantity in the secondary cell wall (Zhou et al., 2017). The degradation of pectin requires the combined action of different enzymes with pectinolytic activity (Zhou et al., 2017). Pectinases (EC 3.1.1.11/6, EC 3.2.1.15/67/82 and EC 4.2.2.10/9/2), as well as cellulases, are considered as key in the bacterial endophytic lifestyle, helping them to colonize, penetrate and spread into plant host tissues (Afzal et al., 2017; Ma 2017).

These enzymes usually act in synergy among them in order to hydrolyze the plant host substrates, namely plant cell walls (Menéndez et al., 2015; Talamantes et al., 2016; Zhou et al., 2017; Nguyen et al., 2018). All of them are gathered in the CAZY database (Lombard et al., 2014) (www.cazy.org), in which this carbohydrate-active enzyme classes are classified in different modules: Glycosyl Transferases (GT), Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE) and Auxiliary Activities (AA).

The breakdown of plant polysaccharides, especially the ones that can be found in the plant cell wall, require a complex and tightly regulated production of lytic enzymes produced by endophytic bacteria (Talamantes et al., 2016; Kandel et al., 2017; Menéndez and García-Fraile, 2017; Menéndez et al., 2017). Bacteria presenting cellulases and other hydrolytic enzymes are usually considered as biocontrol agents (Berendsen et al., 2012; Menéndez and García-Fraile, 2017); however, these enzymes are also related to the entry modes of bacterial endophytes into plants, being involved in the localized breakdown of plant cell walls and in the translocation of several compounds from the outside to the plant apoplast (Santi et al., 2013; Egamberdieva et al., 2017; Liu et al., 2017).

Bacterial endophytes with biocontrol and plant growth promoting properties use to exhibit easily detectable cellulolytic, pectinolytic and xylanolytic activities (Santoyo et al., 2019). Bach et al. examined biocontrol potential of a set of already known PGP bacteria, showing that all of the strains were able to produce hydrolytic enzymes (xylanases, cellulases, pectinases, xanthanases and amylases) in high levels (Bach et al., 2015). On the contrary of what was expected, those strains were not pathogenic on potato tubers. In a study on biocontrol agents against *Verticillium dahliae* were detected several *Pseudomonas* strains with cellulase, one with β -glucosidase and other with xylanase and glucanase activities with potential against this olive tree disease agent (Gómez-Lama Cabanás et al., 2018). However, none of the isolates were positive for pectinase activity. Another study reported that the production of cellulase enzyme in a set of chickpea root endophytes is co-related with the genus affiliation, being the genera *Bacillus*, *Paenibacillus* and *Pseudomonas* the ones holding the highest cellulolytic activities (Brígido et al., 2019). Interestingly, these authors found a higher proportion of isolates when chickpea-growing soils were inoculated with a mesorhizobial strain. These activities were also found in Gram positive plant endophytes both *in vitro* (Trujillo et al., 2015) and *in silico* analysis (Carro et al., 2018), showing the presence of cellulase, pectinase, amylase, and xylanase activities in *Micromonospora* endophytes.

The production of hydrolytic enzymes appears to be necessary in the bacterial endophytic lifestyle (Sessitsch et al., 2012; Walitang et al., 2017; Afzal et al., 2019). The presence of cellulases and related enzymes forming part of cellulose biosynthesis operons are crucial for the successful colonization of different plant hosts (Monteiro et al., 2012; Robledo et al., 2012; Straub et al., 2013). Studies involving NGS were able to identify that genes involved in plant cell wall degradation and invasion are enriched within the genomes from bacterial phytopathogens (Karpinets et al., 2014). These genes are also present in the genomes of many beneficial endophytic bacteria but in less number, codifying for few enzymes with plant cell walls degrading activity, such is the case of the known N_2 -fixing grass endophyte *Azoarcus* sp. BH72 (Krause et al., 2006), the plant growth promoting *Burkholderia phytofirmans* PsJN (Weilharter et al., 2011; Mitter et al., 2013) and the bacterial endophyte of *Miscanthus* biomass grass, *Herbaspirillum frisingense* GSF30^T (Straub et al., 2013). In addition, metagenomic studies showed that genes encoding cellulases, xylanases, cellobiohydrolases, endoglucanases and cellulose-binding proteins were detected in rice root endophytic bacterial

communities (Sessitsch et al., 2012). Houfani et al. compared the associated bacterial communities of several ecosystems (agricultural, garden, desert, among others) and determined functions *in silico* and *in vitro*, highlighting the presence of a higher proportion of genes encoding cellulases, hemicellulases and pectinases assigned to forest soils, in which there is more lignocellulosic materials available for degradation (Houfani et al., 2019).

Thus, beneficial endophytes produce lower levels of plant cell wall degrading enzymes in comparison with phytopathogenic bacteria, which produce deleterious levels of this kind of enzymes. Several members of rhizobia and *Frankia* were described as producers of low amounts of cellulases, hemicellulases and pectinases (Mateos et al., 1992; Jimenéz-Zurdo et al., 1996; Igual et al., 2001). These beneficial endophytes might overcome the plant immune defense pathways, which are triggered in the presence of bacteria (Afzal et al., 2019). In the case of rhizobia, there are several studies showing how the rhizobial strain can erode the apex of the root hair, made of non-crystalline cellulose, by means of the production of cellulase CelC2 in a tightly regulated way and pass through the hole opened on the tip (Robledo et al., 2008, 2012). This rhizobial cellulase elicits some transient responses in the host plant, but those responses are not enough to unleash plant immunity and also, not enough to abort the infection and nodulation process (Robledo et al., 2011, 2018). It has been also reported that the “aggressive” infection of rice roots by *H. seropedicae* Z67 occasionally elicited a localized host defense response (James et al., 2002). This strain also possessed cellulase and pectinase enzymes produced in low levels. It was suggested that the expression of a gene encoding for a pectinesterase (catalysed the de-esterification of pectin into pectate and methanol) is crucial for the association of a non-photosynthetic *Bradyrhizobium* strain to rice roots (Piromyou et al., 2015). The expression of *peces* gene is also transient in response to rice root exudates, suggesting that the levels of this enzyme remained low through the interaction.

Different strains from several *Pseudomonas* spp. are able to produce lytic enzymes, colonized root hairs of olive, barley and rice roots as main point of entry; however, strain PICF7 of *P. putida* appeared to prefer localized spots in the olive root hair tips (Prieto et al., 2011). In *Azoarcus* sp BH72, the expression of the EglA endoglucanase is important for the success of this bacterium in the colonization of rice roots (Reinhold-Hurek et al., 2007). Moreover, *Herbaspirillum frisingense* strains possessed endo-1,4- β -glucanases and α -glucosidase (Straub et al., 2013). Some strains of the diazotrophic bacterium *Gluconoacetobacter diazotrophicus* were able to produce cellulases, hemicellulases and pectinases, bringing the possibility of those enzymes are responsible of the penetration of this bacteria and their subsequent mobility within the root endosphere (Adriano-Anaya et al., 2005). In *B. phytofirmans* PsJN, an endoglucanase, an endopolygalacturonase and other associated plant cell wall degrading enzymes were important in the entry into *Vitis vinifera* roots (Compant et al., 2005). The production of cellulases, pectinases and xylanases is therefore a key step in plant root colonization for many endophytes, producing these enzymes in small quantities; enough for penetration but not for induce plant defences against them.

13.4 Chitinases

Chitinases (EC 3.2.1.14) hydrolyze the β -1,4-glycoside bond of chitin and other N-acetylglucosamine compounds. Their molecular size is between 20 and 90 kDa and most of them belong to the glycosyl hydrolases families 18 (classes III and V) and 19 (classes I, II, and IV) (Kumar et al., 2018). They are produced by a whole range of organisms, from plant to viruses, including bacteria and fungi, and are involved in nutrition, morphogenesis, pathogenic and symbiotic processes (Kasprzewska, 2003). Classically, chitinases have been studied for their role against pathogen attack and systemic defence mechanisms in plants; however, they are also implicated in general growth and development status (Regalado et al., 2000). Velusamy and Kim found a strong correlation between bacterial chitinolytic activity and the lysis of the fungal mycelium under *in vitro* conditions (Velusamy and Kim, 2011). Some genera of endophytic bacteria, such as *Enterobacter*, *Bacillus*, *Micromonospora*, *Streptomyces*, *Burkholderia* and *Stenotrophomonas* were reported with chitinolytic activities and effective against fungal phytopathogens (Tabli et al., 2018; Gasmi et al., 2019). However, Bach et al. did not detect chitinolytic activities in tests performed with isolates, which were positive for the presence of other hydrolytic enzymes (Bach et al., 2015).

Chitinolytic enzymes are able to regulate the colonization process by generating or degrading signal molecules. Nodular factors in rhizobia are basically chitin oligomers (bacterial lipochitooligosaccharides), being chitinases an indirect regulator of rhizobia calls to nodule formation (Cullimore et al., 2001; Cissoko et al., 2018). Lectin-like and chitinase-like proteins have been also proposed as direct receptors for these lipo-chitooligosaccharides, having a direct role in bacteria perception is that is the case. Plant chitinase isoforms have been shown to vary depending on the bacteria that have penetrating the tissues; from one side the recognized bacteria induce regulating chitinases while, from the other side, not compatible or not symbiotic bacteria are thought to induce chitinases that respond to elicitors molecules and trigger plant defence mechanisms (Kasprzewska, 2003).

Plant chitinases have been also related to embryogenesis, ethylene production and tolerance to several biotic and abiotic stresses (Kumar et al., 2018), steps at which PGP bacterial chitinases could also mediate for the induction of beneficial responses. Most of the bacteria chitinases belongs to family 18 of glycosyl hydrolases. Several bacteria chitinases studies have been focused on its utilization as a source of energy, and lately, in its use as biocontrol agents to inhibit the growth of pathogenic fungi. However, it is shown how some mutualistic microorganisms use the production of chitin elicitors obtained after treatment with their own chitinases to prepare their host for the mutualistic relationship (Sánchez-Vallet et al., 2015). Similar to what was observed in pathogens; endophytic microorganisms are subjected to plant defence mechanisms during the first steps of the interaction. Although many of these steps are still undetermined, it is probably that plant endophytes generate effectors to avoid these plant immune responses (Rovenich et al., 2014). The recognition of the plants should be related to their ability to identify chitin decoration or the length of the oligomer generated by pathogens and beneficial microorganisms differentially allowing host discrimination. Nevertheless, the molecular mechanisms underlying these processes are still unknown (Broghammer et al., 2012).

13.5 Lectins

Lectins are carbohydrate-binding proteins, which are distributed as clusters of glycoproteins universally distributed in prokaryotic and eukaryotic cells with implications in insect pest resistance as well as tolerance against other microorganisms (Ingale and Hivrale, 2013). They can be detected in several vegetative organs like bark, bulbs, corms, flowers, fruits, latex, leaves, nectar, phloem sap, rhizomes, roots, stems and tubers. Lectins are extremely specific for their sugar molecules and plant lectins have been shown to possess analogous biological activities as well as chemical properties and are directly implicated in several plant defense mechanisms (Ingale and Hivrale, 2013). It has been also demonstrated that plant lectins are able to recognize the symbiotic bacteria that fix nitrogen in the roots and induce the transport of glycoproteins, sugars and hormones implicated in the symbiotic process (Bohloul and Schmidt 1974).

Between the industrial applications of lectins it can be found the agglutination, toxicity and inhibition or promotion of microorganism's growth. Lectin antibacterial activity is key for plant defense, binding to carbohydrates of the bacterial cell wall or to extracellular glycans. The attachment of lectins from plants to cell wall peptidoglycans shows a strong interaction with muramic acid, and the bacteria are able to recognize these lectins that have been attached to their cell walls, triggering a whole response cascade (Ayoub et al., 1994). Between these recognition molecules, LysM receptor is one of the most studied. This receptor is in charge of the perception and recognition of microbial components including GlcNAc, exemplified by bacterial PGN and fungal chitin (Madsen et al., 2003). In *Arabidopsis*, the AtCERK1 LysM receptor recognizes chitin oligomers triggering the phosphorylation of intracellular kinase domains for plant defense (Petutschnig et al., 2010) and similar is happen in *Oryza sativa* plants (Liu et al., 2012). Recently, the GNA Lectin receptor like protein (LecRLK)-ligand interaction has been identified, being able to detect specifically lipopolysaccharides from *Pseudomonas* and *Xanthomonas* through LORE (Lipooligosaccharide specific reduced elicitation) molecules. However, the downstream signaling responses that induce less susceptibility to the bacterial infection are still unidentified (Ranf et al., 2015).

In addition, lectins are able to stop the flagella mechanisms, preventing the bacterial movement (Xu et al., 2016). In *Azospirillum brasilense*, it was demonstrated that the presence of plant lectins with specificity for N-acetyl- β -D-glucosamine oligomers decelerate the bacteria speed and induce granular colonies as a consequence of the interactions between agglutinins and lectin-binding polymers from bacterial cell wall (Schelud'ko et al., 2009). Some lectins have been also identified as the responsible for the recognition of extracellular ATP (biotic stress), inducing a whole set of responses in the plant structures, as well as activating jasmonic acid signaling and other defense genes (Tripathi et al., 2017). On the other side, the reaction of bacteria to this process has been less studied until now, but if the bacteria are able to inhibit the action of these lectins, they will be more easily penetrating plant tissues. In this line, it has been recently shown how a fungi chitin is able to interact with a rice lectin, preventing the defense mechanism and allowing the fungi infection (Han et al., 2019). Lectins are also accumulated in plant cell nucleus and cytosol after biotic stress, inducing to think they possess a role as defenders or cellular signaling for future invasions (Van Holle and Van Damme, 2018). Another type of lectins implicated in defense are the jacalins, also known as jacalin-related pectins, which are active under biotic and abiotic stress, but also have been shown to be implicated in the development of the plant (Xiao et al., 2015).

Recently, new technologies have generated whole genome sequences from several plant species, allowing the identification of multitude of different lectin sequences. The family is highly complex and its biochemical properties and activities are highly diverse. Only some of them have been functionally characterized, showing implication in several steps of plant-bacteria interactions, both positive and negative, but they are still many unknown functions of these

molecules, existing an enormous potential of plant lectins underrated (Van Holle and Van Damme 2018) and should be the focus of future studies for a better understanding of their implication in plant colonization.

13.6 Lipases

Lipases (EC 3.1.1.3) are enzymes that catalyze the hydrolysis of different lipid substrates and belong to subclass II of the alpha and beta hydrolase superfamily and show a versatile collection of catalytic properties, including aminolysis, alcoholysis, decarboxylation and transesterification. They are essential lipolytic proteins that can be found in great variety of organisms, from prokaryotes to eukaryotes (Lee and Park 2018). Lipases are implicated in a wide range of roles within the plants, including plant germination, signaling processes, plant growth and development, lipid turnover, and stress responses (Matos and Pham-Thi, 2009), amongst others.

Between the defense responses, it has been observed that when microorganisms from air reach leaves found a lipophilic cuticle covering the leaf surface, if they produce lipases for cuticle degradation, plants respond with the release of lipid compounds that act as signal molecules to inform the plant about the microorganism's arrival (Lee and Park, 2018). This liberation of lipids through lipases is a general strategy in plant defense, although the specificity of substrate and cellular responses are highly variable for the different lipases. The glycine-aspartate-serine-leucine (GDSL) lipases have been usually described as plant developers and environmental stress related enzymes. One of these secreted lipases from *Arabidopsis* (GLIP1) increase the expression of plant defense related genes and plant resistance to multiple pathogens (Oh et al., 2005). This lipase, GLIP1, has been also related with directly production of antimicrobial peptides, able to generate spore disruption and hyphae fragmentation of fungi, and dependent of the ethylene pathway (Kim et al., 2013). The capacity of microorganisms to produce ACC deaminase, indirectly regulate ethylene abundance, and would be an important step to avoid plant lipase defenses when PGP bacteria are trying to get access to plant internal tissues. Some microorganisms are also able to produce a type of lipases called cutinases, able to hydrolyze the cuticular polyesters of the plant penetrating this way the host plant. During this process, an increase of permeability has been also observed, allowing the leakage of plant fluids to be used by bacterial for growth (Tang et al., 2007).

The production of lipases by endophytic bacteria has been detected in several analysis, being also related to their ability in plant protection, being the presence of these enzymes in strains with broad antimicrobial activity (Mohamad et al., 2018; Liu et al., 2019). Lipases and other hydrolytic enzymes studied here are related to degradation of fungal cell wall or cell membrane to protect the plants against pathogens, but also with the degradation of extracellular virulence factors and stimulating the plant immune system (Mohamad et al., 2018).

13.7 Proteases

Proteases, also known as peptidases or proteinases, are enzymes that catabolized the breakdown of proteins by hydrolysis of peptide bonds acting at post- and translational levels. It have been described that five main types of proteases can be found depending on the catalytic residue related to the attachment to the substrate bond, including aspartate, cysteine, metalloproteases, serine and threonine (Rawlings et al., 2018). The proteases from fungi and bacteria have an important role in carbon and nitrogen recycling of proteins, including nutritional signals that regulate other process in cascade (Sims, 2006). Moreover, plant proteases have been implicated in the response to microorganisms invasion, triggering the hypersensitive response that induce the programmed cell dead of the invaded cells and the necessary signals to activate defenses in distant areas (Salguero-Linares and Col, 2019). For example, the vacuolar processing enzymes (VPEs), a group of cysteine proteases, react to the bacterial elicitor harpin, inducing the cell dead by the hypersensitive response system in *Nicotiana benthamiana* (Zhang et al., 2010).

Several bacteria have been also shown the ability to produce proteases, which are capable to interact with the products of plant resistance genes. For example, a metalloproteinase produced by an *Erwinia* strain splits the potato lectin produced as plant defense reaction (Feng et al., 2014). Hatsugai et al. (2009) proposed a defense mechanism that consists in the fusion of the central vacuole of the plant with the plasma membrane in the presence of pathogenic bacteria to allow the liberation of antimicrobial hydrolytic enzymes to the apoplast and inhibit bacterial growth in *Arabidopsis*. During this study, they verified that the avirulent infection with the bacteria *Pseudomonas syringae* pv. tomato DC3000 (with AvrRpt2 or AvrRpm1) does not trigger the fusion, and this was responsible of a caspase (type of protease) and proteasome specific inhibitors produced by the bacteria, avoiding the liberation of antimicrobial compounds (Hatsugai et al., 2009). Similar effects have been observed from the other side, existing inhibitors of proteases produced by the plants that stop the activity of these enzymes secreted by the microorganisms and also implicated in microbial growth inhibition (Gomes et al., 2011). As there is an increasing number of studies focused in the analysis of proteases in the

plant-microorganism interactions, it become clear the implication of these enzymes in the communication between both partners, being key signals in recognition process.

13.8 Conclusions and future perspectives

The plant and its microbiome interact between and within them. These interactions could be detrimental (pathogenic microbes), neutral (commensal microbes) or beneficial (mutualists or symbionts). All these associations are going to depend on the exchange of molecules and signaling processes between the partners, definitively, the success of their interaction relies in the communication networks. When microbes interact with plants, trying to colonize and to invade their surface and inner tissues, the plant activate both genetic and metabolic mechanisms of defense to protect themselves and acquire a certain level of host resistance. In these processes, known as gene-for-gene interactions, there is a group of plant genes and/or products encoded by those genes involved in plant resistance, as well as some molecules localized at the surface and other intracellular immune receptors able to recognize microbial derived gene products and generate a response for plant protection. These defense mechanisms are strongly activated when the plant interacts with pathogens. The defense focuses directly on the ways to avoid the pathogen colonization and penetration into plant tissues. When beneficial plant growth promoting bacteria colonize and/or invade plant tissues, the defense response is transient and eventually not enough to abort the colonization and the infection process through the plant cell wall. Hydrolytic and carbohydrate-binding enzymes, namely cellulases, xylanases, pectinases, chitinases, amylases, proteases and lectins, are involved in biocontrol against pathogens and have key importance in those processes related with plant surfaces colonization and invasion through plant cell walls and intercellular spaces. However, most of the effects are still unknown and the implication of degradative enzymes in these processes has not been fully demonstrated. The generation of data by new technologies, including genomics, transcriptomics and proteomics, has increased enormously the knowledge of new hydrolytic enzymes and their roles, as well as their differential expression in colonization and symbiotic processes. We should keep an eye to all the hydrolytic enzymes and their implication on the molecular dialogue at first contact steps. Future studies dedicated to this topic, involving either cultivable or culture-independent approaches, will be of paramount importance in order to achieve a deep understanding of how these bacterial enzymatic toolkits are involved in the bacterial interaction with and within plants.

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Chapter 14

Beneficial role of viruses in plants

Surender Kumar^{1,2}, Reenu Kumari^{1,2} and Vipin Hallan¹

¹Council of Scientific and Industrial Research-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, India., ²Institute of Plant Protection, ARO, The Volcani Center, Rishon LeTsiyon, Israel.

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14.1 Introduction

Viruses are ultra-small, obligatory intracellular moieties often related with disease. Interestingly, they are associated with all life forms. The word “virus” is derived from Latin language, which means “slimy liquid” or “poison”. As per their name and definition, they are considered as harmful which often causes life threatening diseases. A ‘Plant Virus’ is usually a very general term used for viruses found in plants, although they can survive and replicate in some insects, usually acts as their vectors for their transmission. Most of the plant viruses are considered as “obligate intracellular pathogens” as they cause disease in several economic important crops. However, most of the studies are limited only to economically important crops, which were developed by selecting beneficial traits for better production, taste or nutrition like features. In nature, plants live in symbiotic relationship with many microbes and same is true for plant viruses. The most peculiar feature of plant virus symbiosis is that they do not kill their host (with very few exceptions) or we can say that during evolution, host-virus had learned to survive together. Many of the plant and animal viruses are well known to cause disease, which cannot be denied. If we look into history from germ theory (Koch and Pasteur) to viral discovery, it starts from a disease. Ivanovski and Beijerinck identified first virus which was responsible for mosaic disease of Tobacco and named it *Tobacco mosaic virus* (TMV). Later, a number of disease-causing viruses were identified in animals (e.g. Polio virus, Rabies virus, Mesale virus etc.).

As interest grows into these smallest particles and with advancement in genome sequencing technologies, significant efforts were made to characterize these forms upto nucleotide level. Despite of all these efforts, characterization of novel viruses is still a subject of current interest and each year a number of new viruses are being identified in all domains of life, which reflects a lack of knowledge about their diversity. However, in most studies, characterization is limited only to physical properties or up to genome level. This trend is very much similar to bacteria, which were initially considered as pathogens, but now several studies has proved that they are one of essential component for life forms, helping them in infinite ways. Rather such broad studies are lacking in plant virology, where most of the studies are biased, as most of viral characterization starts with symptoms or disease development in important crops. This is one of the possible reasons that we are limited in their diversity knowledge. Also, frequent transport of planting material made these well characterized viruses of important crops worldwide. To get idea of viral population, large metagenomic type studies focusing on different natural niches are required to study the native viral ecology. One of such study involved sequencing of viral population (enriched for viruses by isolating virus like particles) from samples collected from Tallgrass Prairie Preserve (Muthukumar et al., 2009). Results showed that majority of viral sequences did not share significant homology with known viruses. Also, a major portion of total sequence pool does not shared homology

with any known sequence present in database. There remains high possibility that this unmatched sequence pool represents viral sequences that do not belong to any known viruses or viral taxa. Similarly, in another study, double (ds) RNA extracted (to enrichment of viral genome) from samples collected from Tall Grass Prairie Preserve Oklahoma and Area de Conservación Guanacaste in northwestern Costa Rica was used in high-throughput sequencing (Roossinck et al., 2010). Sequencing results showed that 70% of samples showed presence of viruses, however the level of homology is not very high just enough to estimate their family. These two studies clearly showed that our knowledge about viral population is very limited.

With growing interest in viruses, we started understanding their biology. Eventually the information comes in both ways i.e. in terms of host and pathogens and it opens newer era of biology (like pathogenicity, interaction within host, host immune response, genetic engineering, etc.). In this chapter, we are focusing on some of those outcomes, which have been utilized in agronomy or to understand plant biology. When we follow historical achievements, there are many reports of “cross protection” or “vaccination” in past, in which milder strain of virus is used for providing protection against severe strains. All these viral strains naturally exist in host species and were successfully used to control loss caused by severe strains. This phenomenon further led to genetic engineering of plants to provide resistance against harmful viruses. Viruses are known to alter plant biology in many ways and some of these changes had beneficial effects on hosts. Similarly, there are well known group of viruses mainly *Partitiviruses* which do not cause any noticeable disease or loss in crops. With advancement in technology and refinement in knowledge now newer perspective of these viruses starts appearing which can be utilized for benefit of mankind. Beneficial aspect of viruses is a new emerging area of plant virology and limited information is available on it. In this chapter, we are trying to shed light on some of the useful aspects of viruses which are or can be utilized in agriculture.

14.2 Virus infection improves host tolerance to various stresses

Being sessile in nature, plants face a number of biotic and abiotic stresses in field conditions. High temperature and soil water deficit (WD) are the major abiotic stress causing changes in host growth and productivity (Zhang and Sonnewald, 2017). Virus-plant being in mutualistic association, changes the adverse effect in different ways by communicating and signaling host defense. To study the effect of viral infection during WD conditions, Bergès et al., 2018 infected nine wild accessions of *Arabidopsis thaliana* (L.) Heynh (*Brassicaceae*) showing differential responses in WD conditions, with *Cauliflower mosaic virus* (CaMV; *Caulimoviridae*). Symptom development and virus transmission in WD and well-watered (WW) plants showed variable response in different accessions and it depends mainly of plant genotype. Interestingly, viral infection within WD plants was slower as compared to controls (WW) plants.

In another study, it was observed that RNA viruses improve plant tolerance to abiotic stress, indicating a conditional mutualistic relationship which helps host to survive in adverse conditions (Xu et al., 2008). Four different viruses namely *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) and *Tobacco rattle virus* (TRV) were inoculated on beet, pepper, watermelon, cucumber, tomato, zucchini, rice, tobacco, *Chenopodium amaranticolor*, *Nicotiana benthamiana* and *N. tabacum*. After infection, drought stress or WD was imposed. It was found that CMV infection improved the drought tolerance in the inoculated plants and enhanced freezing tolerance in beet plants. In *N. benthamiana*, a common host for all these viruses showed 2–5 days delay in appearance of drought related symptoms as compared to the control plants. Metabolite profiling by gas chromatography–mass spectrometry (GC–MS) of the infected and control plants showed a significant increase in the accumulation of anti-oxidants and osmoprotectants in virus infected plants, helping the plants to tolerate drought stress.

Similarly, 2b protein of CMV is known to increase drought tolerance in *A. thaliana* (Westwood et al., 2013). *A. thaliana* plants infected with Fny-CMV and its mutant who lacks 2b protein, showed significantly higher water contents than control/mock-inoculated plants and does not displayed wilting or drought related symptoms even after 8 days of drought stress. However, CMV mutant (2b) infected plants showed wilting and drought related symptoms, like the control plants. This study clearly showed that 2b protein of CMV plays crucial role in plant survival during drought conditions. Further to confirm the role of 2b, transgenic *A. thaliana* plants expressing 2b were generated and these plants also showed increased drought tolerance. A significant water loss was observed in control plants (90%) after drought treated whereas in transgenic plants showed water loss only up to 50%. These studies showed that viral infection increases the host tolerance to abiotic stresses, which ensure their long term survival and is beneficial for host and virus.

A classic example of this host-pathogen survival is Pigeonpea plants. Pigeonpea plants infected with sterility mosaic disease (SMD), caused by two different emaraviruses (named *Pigeonpea sterility mosaic virus-I* and *-II*; PPSMV-I and -II) showed prolonged survival of their host as compared to the healthy plants (Kumar et al., 2017a). Márquez et al. (2007) provided evidence of a very unique three-way symbiosis, which provides high temperature tolerance to their host.

Initially, they found a symbiotic association between an endophytic fungus *Curvularia protuberata* and *Dichanthelium lanuginosum*, a tropical panic grass growing in geothermal soils of Lassen volcanic (LVNP) and Yellowstone (YNP) national Parks (Redman et al., 2002). This association allowed both plant and fungi to survive at very high temperature (up to 65 °C). But in non-symbiotic conditions, both plant and fungus failed to grow at 38 °C, demonstrating that this association provides thermal tolerance. Later, they isolated a new bipartite, double stranded RNA (dsRNA) mycovirus from the fungus named *Curvularia thermal tolerance virus* (CThTV; Márquez et al., 2007). The virus was found in high titer among wild type isolates of *C. protuberata*. To study the association between fungus and virus, they further produced virus free cultures of fungus. Plants of *D. lanuginosum* were inoculated with virus infected fungus and without virus and heat stress was induced. In this study, it was found that only plants infected with fungus containing virus survived at 65 °C and others died, which clearly showed the benefit effects of each species to their hosts (Márquez et al., 2007). These studies showed that viruses provide advantages to their hosts in adverse conditions.

14.3 Gene transfer between viruses and hosts

A number of pararetro- and non-retroviral (mainly *Partitiviridae*) viruses' sequences are known to exist in the plant genome. Similarly, viruses are known to harbor some plant genes, for e.g. ubiquitin, chloroplast and heat shock proteins. Few of these studies were discussed in this section. Are these sequences results of endogenization of viral/host sequences as a result of mutualistic affair between host-virus or these viruses somehow originated from plant genome during evolution? This is still a question of debate as solid evidences in favor of any one is still lacking, however growing evidences showed different roles of these sequences in plant genomes.

Plant pararetroviruses belongs to family *Caulimoviridae* and contains double stranded (ds) DNA genomes and like retroelements they use reverse transcription for their replication. Recently, endogenous pararetroviral sequences (EPRVs) are found in number of plant species like tobacco (*Nicotiana tabacum*) and other *Nicotiana* species, tomato, potato, banana, petunia and rice etc. Staginnus et al. (2007) identifies EPRV sequences in five different cultivars of tomato, which showed a varied similarity with coat protein (CP), cell-to-cell movement protein (MP), polyprotein (POL) and transactivator protein (TAV) of Tobacco vein clearing virus (TVCV), suggesting their common origin. Interestingly, all of these protein coding regions are non functional or translationally defective like in *Nicotiana* and are present in all chromosomes. Hybrid generated from cross between these tomato cultivars does not show any reactivation of virus as observed in banana. Tomato plants harboring *LycEPRV* showed increase in small interfering RNA (siRNA) population, when challenged with unrelated viruses (like PVY). Based on this it was speculated that they may suppress the pathogenicity of endogenous pararetroviruses by RNA silencing mechanism. These results are different from other EPRVs like found in Banana belonging to *Banana streak virus* (BSV), in which virus showed reactivation by interspecific reactivation (Ndowora et al., 1999).

Advancement in next generation sequencing technologies led to generation of enormous genome-based sequence data, which is helpful to understand plant biology in better ways. Using the available plant genome sequence information Chiba et al. (2011) identifies endogenized non-retroviral RNA virus sequences (NRVSSs) in plant chromosomes, which are derived from plus-, negative- and double-stranded RNA viruses. The most common NRVSSs were coat protein (CP) genes of the family *Partitiviridae*. These viruses contain bi- and tri-segmented dsRNA genomes and able to infect plants, fungus and protozoans (Nibert et al., 2014; Kumar et al., 2017a,b). Recently, Kumar et al. (2017b) identifies a unique tripartite cryptic virus named as *Arhar cryptic virus-1* (ArCV-1). Its RNA-1 encodes for RNA dependent RNA polymerase (RdRp) and it shares 71–72.4% similarity with other cryptic viruses. RNA-2 and 3 encodes for two different coat proteins (CP) and they shared 39% and 40% similarity with other cryptic viruses. Interestingly, these dsRNAs RNA-1, -2 and -3 shares 64%, 34% and 61% homology with unknown proteins *Picea sitchensis* (ADE76327), *Cucumis melo* (XP008459635) and *Vigna angularis* (KOM46435 and XP017431256) respectively. In addition to this, another viral genomic segment named as dsRNA-2A, which showed homology to CP gene (maximum 36% with *Raphanus sativus cryptic virus-3*) of bipartite partitiviruses was identified in this study. The dsRNA-2A also showed 34% sequence identity with ATP-binding cassette (ABC) transporter F family member-1 (KHF98106) of *Gossypium arboreum* (tree cotton). These plant species are not known to host any cryptic viruses.

14.4 Plant viruses in cross protection

The phenomena in which a mild viral strain (not causing yield loss) is used to prevent disease caused by related severe viral strain is called cross protection. It is a form of acquired immunity gained by host species, which prevents or blocks the multiplication of other related viruses through unknown mechanism, unrelated to RNA silencing. The strains used

to provide immunity is known as “protector strains” whereas severe strain causing disease is called “challengers” or “secondary infections”. This phenomenon was first described by McKinney (1926) in two genotypes of *Tobacco mosaic virus* (TMV, Genus *Tobamovirus*). One of TMV strain induces light green mosaic symptoms whereas other strain causing severe yellow mosaic symptoms. Tobacco plants infected with green mosaic strain remained protected against yellow mosaic symptoms on subsequent inoculation with TMV yellow mosaic strain. Later this phenomenon was observed in many viruses belonging to different taxonomic groups. The first commercial use of this phenomenon was reported with *Citrus tristeza virus* (CTV: Genus *Closterovirus*).

Citrus crop is well known to be infected by CTV around world. CTV is one of the most complex and largest known plant viruses, which belongs to family *Closteroviridae* and is phloem limited virus (Dawson et al., 2013). Naturally CTV is restricted only to citrus and its relatives. It causes two types of diseases in citrus (i) quick decline disease in plants propagated on the sour orange rootstock, which results from a virus-induced graft incompatibility between the scion and rootstock and (ii) stem pitting disease which irrespective of rootstock affects many citrus varieties and causes reduced plant growth, vigor, fruit size and quality (Folimonova, 2013, Lee and Keremane, 2013). As quick decline disease is rootstock specific using resistant/tolerant/different rootstocks would overcome this problem but this is not a case with stem pitting disease as it is not limited to rootstocks. Using mild strains to provide protection against severe strains became prominent strategy to decrease the loss caused by CTV and is successfully used in many citrus growing regions/countries. However, cross protection needs careful selection of protective strains. In Brazil, 80 million Pera sweet orange trees are protected by cross protection from severe CTV strain. In order to find a protective strain, a number of sweet orange, lime, and grapefruit were surveyed and 45 mild isolates were selected for field trials, which involves near to 2300 trees. Out of the 45 isolates, six isolates were found satisfactory for further use in cross protection which includes three for Pera sweet orange, two for Galengo lime and one for Ruby Red grape fruit (Costa and Müller, 1980). However, isolates of Pera cv. does not provide protective covering in Galengo or in Ruby cvs and same in vice-versa, which reflects a very high specificity is required to provide resistance against severe strains and it needs a deep study to understand this interconnection. Similar approach had been opted in other parts of world for protection of citrus against CTV. This is one of the first successful uses of cross protection phenomena in commercially booming citrus industry.

Another important crop in which cross protection phenomenon was used is papaya. *Papaya ring spot virus* (PRSV) causes papaya ringspot disease in this crop. PRSV belongs to genus *Potyvirus*, family *Potyviridae* and is transmitted by aphids in a nonpersistent manner (Purcifull et al., 1984). PRSV infection greatly reduces the quantity and quality of the fruit. Due to the impact of PRSV disease, farmers in different parts of world abandon the cultivation of papaya. Using chemical mutagenesis, two milder strains named as PRSV-HA 5-1 and PRSV-HA 6-1 were synthesized from severe Hawaii strain (PRSV-HA). These mild strains were used in different parts of Taiwan and Hawaii to protect against PRSV disease with varied success (Gonsalves and Garnsey, 1989). In contrast to this, a naturally occurring mild strain from India, showed more promising results in field trials and glass house experiments (Ram et al., 2006).

14.5 Viruses and their role in plant biology

Viruses act as catalyst to understand host biology specially to understand host defense network which leads to development of strategies to counter pathogenic viruses. These studies lead to understand the host innate defense system i.e. RNA silencing or RNA interference (RNAi) in a better way. Also, studies on viruses leads to development of virus-based tools by modifying viral genome i.e. virus induced gene silencing (VIGS). Both of these i.e. RNAi and VIGS contribute significantly to understand the plant biology and leads the way to develop new strategies for controlling pathogenic viruses or to develop better yielding cvs by genetic engineering. Both these are reverse genetics approaches used to study functional genomics. In this section, we are mainly focusing RNA silencing and VIGS system and how this knowledge is contributing to counter pathogenic viruses and to develop better varieties by understanding gene functions.

14.5.1 RNA silencing

RNA silencing or RNA interference (RNAi) is a conserved innate defense mechanism, by which plants degrade the exogenous or viral RNA molecules by sequence complementary manner. RNAi components are triggered by double stranded RNA (dsRNA) or hairpin structured RNA (hpRNA) molecules and these are processed by Dicer (DCL) into 20–24 nucleotide (nt) RNA molecules known as small RNAs. These small RNA duplexes are then loaded into RNA-induced silencing complex (RISC) and these guide RISC to cleave their complementary regions on single-stranded RNA (Baulcombe, 2012; Wang et al., 2012; Guo et al., 2016). There are four RNA silencing pathways in plants namely, (i) the micro (mi) RNA pathway (play a crucial role in the plant development by repressing or expressing the

regulatory genes), (ii) the small interfering (si) RNA-directed RNA degradation pathway (it leads to degradation of endogenous or exogenous long dsRNA or hpRNA, playing major role in plant development, stress response and in plant antiviral defense), (iii) trans-acting small interfering RNA (tasiRNA) pathway (having role in plant development and disease resistance) and (iv) RNA-directed DNA methylation (RdDM) pathway (plays important role in silencing of transposons and repetitive DNA elements). In 1998, potato resistance to PVY was successfully generated using a hairpin dsRNA construct of PVY HCPro (Waterhouse et al., 1998). In this study, a hairpin construct containing sense and antisense strand of PVY HCPro was generated. Once the presence of exogenous dsRNA is sensed by RNAi machinery components, it was by RISC cleaved into 21–24 nt long siRNA duplexes. These siRNA duplexes were used by plant RNA dependent RNA polymerases (RDRs) as template for synthesis of dsRNA and a constant signal of siRNA was maintained within host. When plants expressing this HCPro-hp construct are challenged with PVY, host silencing machinery recognize the target i.e. HCPro on viral RNA and cleaved it into siRNA, making virus inefficient to cause infection. This is first report in which RNAi was used to generate defense against viruses. After this RNAi based resistance was developed against many viruses or viroids. Presently, RNA is not only deployed to generate resistance against pathogens, it is also used to understand functional genomics of plants for e.g. development of seedless fruits in tomato by suppressing the function of auxin response factor 7 (ARF7); modifying color and scent of commercially important ornamental flowers; in enhancement of nutritional values by increasing the level of important secondary metabolites like carotenoids and flavonoids and in development of industrial valued products (reviewed in detail by Guo et al., 2016). It is clear from these examples that knowledge gained by studying this important aspect of host defense against viruses significantly contributes to understand functional biology of plant and in crop improvement.

14.5.2 Virus induced gene silencing (VIGS)

VIGS is a modified RNA silencing technique, in which modified virus genome is used to generate dsRNA for transient silencing assay for functional genome study. This method complements RNA silencing, but it does not require generation of stable transgenic lines. Here, viral genome is modified in such a way that they can accommodate foreign or plant genes and their pathogenicity determinate regions are compromised so that they do not induce pathogenicity. VIGS have been widely used for functional gene studies in many plant species and a number of DNA and RNA viruses are modified as viral vectors. These viral vectors are able to infect wide range of plants species (both monocots to dicots) ranges from simple *A. thaliana* to complex tree species like apple and pear (Burch-Smith et al., 2006; Sasaki et al., 2011). One of most widely used vector is *Tobacco rattle virus* (TRV), which infect wide range of plant species constructed by Liu et al. (2002) is discussed in detail (Liu et al., 2002). TRV is a bipartite single stranded positive sense RNA virus, belonging to genus *Tobravirus*, family *Virgaviridae* and transmitted by nematodes. RNA-1 is approximately 6.7 kb long which encodes for replication, movement and a cysteine rich protein. RNA-2 is approximately 3.6 kb long and encodes for coat protein and two non-structural proteins 2b and 2c. In absence of RNA-2, RNA-1 alone is capable of replication and systemic movement. To develop it as viral vector Liu et al., 2002 cloned full length cDNA of RNA-1 into T-DNA vector containing 35 S promoter named as pTRV-1. To facilitate cloning of foreign or plant genes multiple cloning site (MCS) were introduced in place of 2b and 2c proteins of RNA-2, named as pTRV-2. After cloning of foreign gene in pTRV-2, these recombinants were infiltrated to plants by using *Agrobacterium tumefaciens*. Using *phytoene desaturase* (PDS) gene of *N. benthamiana* as a visual marker, authors successfully showed the silencing of the PDS gene as the infiltrated plants displayed systematic photo-bleached (white) phenotype instead of green (Liu et al., 2002).

14.6 Conclusion

Viruses are one of ubiquitous form present in all life forms. The studies compiled in this chapter clearly showed that they play beneficial role in plant life. However, these kinds of studies are limited, as viral potential is not yet exploited. Above mentioned studies clearly showed various positive impacts of the viruses in different crops. Alteration in functional biology of host by viruses is helping plants to survive in adverse conditions. Better understanding of these phenomena could help us in future to mitigate the effects of frequently changing environmental conditions for agriculture. Already by understanding the viral diversity, phenomena like cross protection is applied in agronomical crops to control pathogenic viruses. The knowledge gained by studying host defense response also contribute significantly to counter pathogens and in host functional genomics. Tools like VIGS are playing significant roles in functional biology. The major bottleneck in plant virology is lack in available knowledge of viral diversity in nature as most studies focused mainly on disease and this needs to be addressed soon.

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Chapter 15

Microbial phytases in plant minerals acquisition

Gurdeep Kaur

Department of Agricultural Sciences, Chandigarh University, Mohali, India

Chapter outline

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15.1 Introduction

As the total population is expanding step by step, there is requirement for a lot of agrarian sustenance harvests to meet the prerequisite of developing population. Harvests need a few supplements to achieve their most extreme potential yield. Phosphorus after nitrogen is next fundamental mineral nutrient for plant growth, development as well as improvement (Bagyaraj et al., 2000). Approximately 98% soil has insufficient supply of accessible phosphorus (Hansan, 1996), prone to incite inadequacy of this mineral. At the point when P as compost add up to the dirt, it gets fix in soil. The phosphate solubilising microbes present in soil solubilize insoluble form of phosphorus and make it available to the plant system (Hilda and Fraga, 1999).

Just 0.1% of the complete P exists in a solvent structure in soil accessible for plant take-up in view of its fixation into an inaccessible structure (Zhou et al., 1992). Phosphorus is taken up from the dirt as solvent orthophosphate particles $\text{H}_2\text{PO}_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and availability of orthophosphate particles to the plants is in sequence of $\text{H}_2\text{PO}_4^{-1} > \text{HPO}_4^{-2} > \text{PO}_4^{-3}$. The kind of orthophosphate particle existing in soil is reliant on topsoil reaction. Generally at decreasing pH of 4–5, orthophosphate typically present in form $\text{H}_2\text{PO}_4^{-1}$ particles. While expanding pH, first of all HPO_4^{-2} particles are formed then convert to PO_4^{-3} as the dirt response ends up antacid. Expansive measure of P connected as manure goes into the stable pools by precipitation response with exceedingly receptive Al^{3+} and Fe^{3+} in acidic soil and Ca^{2+} in calcareous or typical soils (Gyaneshwar et al., 2002). It is therefore that dirt P winds up bound and accessible level of P must be enhanced in all agricultural soils by including substance phosphorus fertilizers, that is not only speak to a noteworthy expense for rural creation yet in addition force unfriendly ecological effects on and large soil, freshwater and marine assets (Tilman et al., 2002). The rehashed and imprudent utilizations of substance P manures, prompts the loss of soil fruitfulness (Gyaneshwar et al., 2002) by irritating microbial assorted variety, and thus diminishing yield of harvests. Utilization of chemical phosphatic fertilizers has turned into an expensive illicit relationship and there is requirement of elective sources. The rock phosphorus deposits were found as a valuable alternative of chemical P fertilizer (Reddy et al., 2002). Utilization of rock phosphates in place of chemical fertilizers has gotten huge interest. It is evaluated in India that there are about 40 million tons of phosphatic rock deposits are present and this material ought to give low cost phosphate fertilizers for agriculture production (Halder et al., 1991). But RP isn't easily absorbed by plant with a pH more prominent than 5.5–6.0 and, notwithstanding when optimum conditions are available, still there is low

crop production as compared to those obtained with soluble phosphate (Khasawneh and Doll, 1979). As of late, microbial items have been in more consideration, as they have huge rural esteem. Numerous metabolites are produced by microorganisms few of them have ability to solubilize insoluble form of phosphate. Phosphorus is major macro element for agriculture production but as observed by many scientists that world assets of economical rock phosphate may exhausted by 2050 (Vance et al., 2003). The absence of economical phosphorus becomes a dangerous emergency alarm in horticulture (Abelson, 1999). Improvement in plant potential for P uptake and usage might have some positive effects on agronomy and nature (Rodríguez and Fraga, 1999; George et al., 2009; Wasaki et al., 2009; Richardson et al., 2009). Approximately 30–80% of soil total phosphorous is present in organic form play a vital role in soil P cycle (Tarafdar and Gharu, 2006).

The phytates are overwhelming type of natural phosphorus, which is 60% of total soil natural phosphorus. These phytate are ineffectively used by agricultural crop plants (Mudge et al., 2003). For plant utilization phytate needs to be converted into simple P form (George et al., 2009; Wasaki et al., 2009). Phytases converted into simple form of P by dephosphorylation process with the help of phosphatases enzyme for example phytases, prior to absorbed by the plant system (Yadav and Tarafdar, 2007a,b; Richardson et al., 2009). Plant roots secrete some metabolites that are used by soil microorganisms as promptly accessible carbon source for their growth and development. Notwithstanding, microbes in rhizosphere need their close association in roots of the plant system for the fulfilment of their nutrient requirements such as phosphorus. Due to presence of phosphatases hydrolysis of organic compounds take place in rhizosphere (Yadav and Tarafdar, 2007a,b). Due to the lack of satisfactory levels of extracellular phytase in the rhizospheric soil majority of the plant species are not able to use phytate as organic source of phosphorus. Exogenously add up of phytases (Singh and Satyanarayana, 2010), molecular expression of phytase gene from *Aspergillus niger* in transgenic plants (Richardson et al., 2001a), the plants will be able to take-up phytate phosphorus. In this manner, if phytase are available to plant in rhizospheric zone may help in improvement of plant system under field conditions. Rhizospheric microorganisms showed phytase activity for example, *Sporotrichum thermophile* (Singh and Satyanarayana, 2010), *Discosia* sp. FIHB 571 (Rahi et al., 2009), *Pseudomonas* sp. (Richardson et al., 2001b) and *Bacillus amyloliquefaciens* (Idriss et al., 2002) were observed to play important role in plant growth and development. Plant growth promoting bacteria (PGPR) are free living microbes in rhizospheric soil and they showed gainful impacts on the plant growth (Kloepper et al., 1988; Patel et al., 2010). The PGPR improve plant growth and development by production of plant growth promoting substances (Steenhoudt and Vanderleyden, 2000) or because of the improvement of nutrient accessibility (Nautiyal et al., 2000). It has been observed that phytates generally converted into insoluble phytates and fixed to the soil particles make them less assessable to the crop system and microorganisms in soil (Hayes et al., 2000). This fixed form of phytates can be making accessible to the plant with the help of phytases enzyme and by organic acids (Hens et al., 2003; Singh and Satyanarayana, 2010). The main mechanism behind the release of soluble form of phosphorous from insoluble phytases and from soil complexes is organic acid production from root exudates that perform chelation reaction that help in release of soluble phosphorous from metal cations-phytase complexes (Tang et al., 2006; Singh and Satyanarayana, 2010). Adams and Pate (1992) demonstrated the theory by taking example of white lupin (*Lupinus albus* L.) have more efficiency to produce organic acid as compared to other species and showed enthusiastic development with sodium phytate when they use it as main source of P. The mechanism of conversion of insoluble phosphates into dissolvable structures through phosphate solubilizing bacteria is acidification, chelation and exchange reactions (Gerke et al., 2000). Phytases and Acid phosphatases discharged by phosphate solubilizing bacteria likewise have an imperative job in P-solubilization (Relwani et al., 2008). This technique not just makes up for greater expense of manufacturing fertilizers in industry yet in addition solubilize the fertilizers added to soil (Reddy et al., 2002). The changing situation requests to adjust the procedures/ways to deal with proselyte an inaccessible portion of soil P into accessible structure for plant take-up. Among the different procedures known for this reasons, utilization of explicit microflora is considered as a standout amongst the most productive intends to solubilize insoluble soil P. Microbial intervened management of phosphorus is a cost effective affair and environment friendly approach for manageable improvement of crop production. Phosphate solubilizing microbes through different processes of solubilization and mineralization can change over inorganic and organic soil P separately into the bio-available structure encouraging take-up by plant roots (Khan et al., 2009). Because of this action, P-solubilizing microorganisms assume a vital job in enhancing phosphorous availability to crop system and permit feasible utilization of phosphatic substances present in soil. It has been also accounted that amendment of rock phosphate along with phosphate solubilizing microbes provide more sustainable results, since it can build P accessibility (Reyes et al., 2002) and it helps in improvement of RP amended soil biological and physiochemical properties (Caravaca et al., 2004; Kaur and Reddy, 2014). The inoculation of microbes as biofertilizers in field condition is most efficient alternative of chemical P fertilizers. Main consideration of this chapter is phytase and their role in insoluble P solubilization in organic phosphorous sources. In addition to this we

will also talk about different biology techniques help in expand soil phosphorus accessibility using biofertilizers fit for discharging microbial phytases into rhizosphere.

15.2 Organic P in soil

There are number of inorganic form of phosphorous are present despite of that a big division of organic phosphate is also present in soil (Giles et al., 2011; Menezes-Blackburn et al., 2013). It has been estimated that out of total P of soil 30%–80% is present in organic form (Harrison, 1987). Phytate is considered as 50–60% of all organic types of phosphorous available in soil (Turner et al., 2002). Phytate is a moderately steady complex, generally present in precipitated or as immobilized complexes. This type of precipitates formed chelation with metal cations and are not easily accessible to plant and microbes (Singh and Satyanarayana, 2010). Phytates are present in abundant form in soil but still these are not available to plants because low phytase secretion efficiency of plant roots and less availability of phytases in plant root system (Richardson et al., 2001a). Phytases enzymes release inorganic form of phosphorous from phytates. Numerous microorganisms especially fungi and bacteria are responsible for production of extracellular phytases in soil and rhizosphere these phytase of microbial origin have high efficiency to convert phytates to inorganic phosphorous and make it accessible to the plants (Mukhametzyanova et al., 2012; Akhmetova et al., 2013). The ability of microbe to produce phytases will used in making transgenic plant with their genetic modifications to produce extracellular phytases and such type of plant will make available insoluble P in soil and able to used as nutrient which will directly help in enhancing crop production (Fu et al., 2008). What's more, such hereditarily adjusted plants could possibly help take care of environmental issues by diminishing phytate aggregation in soil and water (Gontia et al., 2012). A few incredible research papers are available providing literature on microbial phytases and are highly suggested (Giles et al., 2011; Gontia et al., 2012; Menezes-Blackburn et al., 2013).

The myo-inositol, scyllo-inositol, chiro-inositol and neo- inositol are generally present forms of phosphorylated inositols in soil out of which myo-inositol hexakisphosphate is extensively present as compared to others, other three are present in higher to lower concentration in sequence of scyllo-inositol > chiro-inositol > neo- inositol not that much familiar in biological system (Turner, 2007). Most of the plants and soil microorganisms are able to synthesize a number of soil inositol phosphates. Undoubtedly, different plant seeds and some of eukaryotes contain myo-inositol hexakisphosphates. In aleurone layer of barley seeds scyllo inositols are found in monophosphate structure. A little amount of dchiro - inositol hexakisphosphates are available in needles of gymnosperms and in angiosperm leaves (Turner, 2007). Phytate found as principle source of phosphorous in plants, form approximately 30% P-fractions in roots, 80% in seeds and grains (Lott et al., 2000). During seed formation, phytate store as P-form and again accessible to the plant at the time of seed germination by means of natural phytases (Greiner et al., 1995). Seed phytate are generally stored as globular incorporations in aleurone layer and in embryo vacuoles. In case of maize 80% of phytate is available in embryo (O'Dell et al., 1972). Seed maturation and sprouting is directly depend on pH, temperature, metal cation concentration that help in transformation of phytate in seed to absorbable form (Reddy et al., 1989).

15.3 Mechanisms of P solubilization by PSMs

Phosphate solubilizing microorganisms are responsible for phosphate solubilization through a plethora of mechanisms time to time reported by scientists. Most important mechanisms are organic and inorganic acid secretion and proton exchange theory (Roos and Luckner, 1984).

15.4 H⁺ excretion

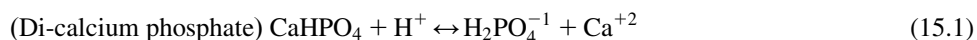
In supply of different nitrogen sources, microorganisms releases H⁺ due to intake of cations. In case of fungi it is well reported that H⁺ is produced during intake of NH⁴⁺ ions (Banik and Dey, 1982; Asea et al., 1988). It has been reported that RP solubilization increased while utilizing a NH⁴⁺ as opposed to a NO³⁻ source of N (Whitelaw et al., 1999). Similarly there is decrease in pH and high rate of titratable acidity by using NH⁴⁺ as nitrogen source. A number of N sources were tried by Vora and Shelat (1998) and he observed that ammonium sulfate enhance the rock phosphate solubilization for bacterial species, *Bacillus circulans*, *Bacillus brevis*, and *Bacillus coagulans*. In certain microbes intake of NH⁴⁺ and production of H⁺ ions is by all accounts the sole system for P solubilization.

15.5 Organic acid production

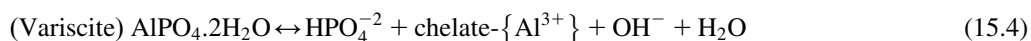
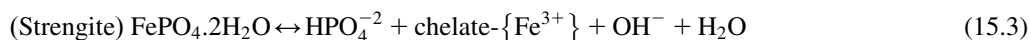
Different types of organic acids are produced by microorganisms in different concentrations. Microorganisms are known to produce organic acids in varying concentration and types. Commonly reported organic acids produced by microorganisms incorporate gluconic acid, citric acid and oxalic acid (Richardson et al., 2001a). Rhizospheric phosphate solubilizing microorganisms produce various kinds of organic acids that are successful in solubilization of insoluble P in soil (Marschner, 1997). It has been observed by Kim et al. (1997) that rock phosphate solubilization by phosphate solubilizing rhizobacteria *Enterobacter agglomerans* was mainly due to the organic acid production yet different mechanisms may involved. In laboratory conditions phosphate solubilizing rhizobacteria secrete organic acids and decrease the pH of the culture medium. Organic acids produced by microbes are generally result of their metabolism, oxidative respiration or due to fermentation of glucose or other carbon sources. Different organic acids produced by PSRB in culture media basically observed are oxalic acid, gluconic acid, citric acid, lactic acid, tartaric acid, aspartic acid, lactic acid (Kim et al., 1997; Bar-Yosef et al., 1999).

In case of Gram-negative bacteria, Goldstein (1995) depicted that inorganic phosphate solubilization is directly related to the glucose dissimilation through oxidation. The study of mineral phosphate solubilization mechanism declare that direct oxidation pathway is response for P solubilization by number of bacteria and biochemical pathways involved in it are Embden-Meyerhof and Pentose phosphate pathways.

By inoculation of phosphate solubilizing rhizobacteria in alkaline soil, rhizospheric pH will decreased by acid production and help in solubilization of apatites and calcium phosphate. The increase in concentration of H^+ ions in the reactants of the reaction (15.1) and (15.2), help in sequestering of calcium from calcium phosphate.



Solubility of phosphate in acid soils is control by variscite and strengite minerals (Lindsay, 1979). Organic acid produced by microbes do chelation with Al and Fe ions, and the reaction 15.3 and 15.4 continue to one side. In any case, this point is in light of the fact that the decrease in soil pH may likewise help in solubilization of other iron and aluminum minerals that would reprecipitate again phosphates to form newly strengite and variscite (Lindsay, 1979).



15.6 Acidification

Organic acids produced in culture medium by microorganisms, carboxyl and hydroxyl ions of organic acids do chelation with cations and decrease the pH help in solubilization of insoluble phosphorous. Direct oxidation pathway involved in release of organic acid in periplasmic space and drop the pH and do acidification of cell and surrounding area of microbes as a result bound form of P will be released (Zhao et al., 2014).

15.7 Chelation

Other mechanism of P solubilization though organic acid is chelation reaction (Whitelaw, 2000). Oxygen containing hydroxyl and carboxyl groups of organic acid are capable of forming complexes with cations such as Ca^{2+} , Fe^{2+} , Fe^{3+} , and Al^{3+} , these cations frequently make bound with phosphate (Kucey, 1983). Organic acid anions make complexes with cations on the mineral surface, and loosen the cation-oxygen bonds of the mineral structure and release the cations to solution (Welch et al., 2002). Results from an examination by Kpombrekou and Tabatabai (1994) demonstrated that solubilization of rock phosphate by organic acid is basically influenced by type and position of functional group and chemical nature of organic ligand. Narsian and Patel (2000) further reported that functional group of chelators especially carboxylic and phenolic hydroxyls of known chelators including EDTA, DTPA, NTA, aluminon, and oxine effect the solubilization of rock phosphate. It has been observed that rock phosphate solubilization will be increased with increased number of carboxyl group on an acid (Xu et al., 2004). Tri carboxylic acids (citric acid) more readily chelate with Ca^{2+} as compared to di-carboxylic acids such as malic and tartaric acids (Whitelaw, 2000). There will be increase in RP solubilization with an expanded number of hydroxyl groups of an organic acid anion to break up RP (Kpombrekou and Tabatabai, 1994). Numerous phosphate solubilizing microorganisms produce 2-keto gluconic acid as

metabolic product is incredible chelators of calcium and extremely compelling in solubilization of hydroxyapatite, fluorapatites and aluminum phosphate (Duff et al., 1994).

15.8 Ligand exchange reactions

Gerke et al. (2000) did investigation with four soils of fluctuating pH, CaCO_3 and organic C substance and observed that the soil with addition of citrate more P was assembled as compared the conditions when large changes in pH. This was due to the ligand exchange between the citrate and the phosphate adsorbed to the Fe- and Al-sites rather than disintegration from Ca-P precipitates. P release by citric acid is effective only when it is adsorbed at levels more prominent than 10 mol g^{-1} soil; anyway beneath the basic esteem, citric acid won't out-compete phosphate for soil adsorption sites (Gerke et al., 2000).

15.9 Mineralization of organic phosphorus

Some specific enzymes are produced by Phosphate-solubilizing microorganisms that help in solubilization of organic form of phosphorous into inorganic form (Rodríguez and Fraga, 1999). Three type of enzymes help in solubilization of organic form of phosphorous (1) Non specific phosphatases, which perform dephosphorylation of phosphoester (2) Phytases, which specifically cause P release from phytic acid, and (3) phosphonates and C-P lyases, perform C-P cleavage in organophosphonates. Main action of p solubilization is due to the acid phosphatases and phytases enzymes as a result of their sufficient substrates quantity in soil (Rodriguez et al., 2006).

Solubilization of Organic phosphate is likewise also known as mineralization, and it happens in soil rich with natural organic phosphorus. Organic phosphorus is 4–90% of total soil phosphorus. Nucleic acids, phospholipids, and sugar phosphates are productively breakdown into simple form but phytic acid, polyphosphates and phosphonates are breakdown slowly the reason is degradation ability of organic phosphorous compounds mostly depends on biochemical and physiochemical properties of that particular molecule (McGrath et al., 1995; Ohtake et al., 1996). This breakdown of organic molecules into absorbable from through mineralization process take place with the help of few phosphatases (phosphohydrolases). These phosphatases enzymes do dephosphorylating reactions include the hydrolysis of phosphoester and phosphoanhydride bonds. The phosphatases are grouped into acid phosphatases and alkaline phosphatases according to their efficiency of dephosphorylating reactions in acidic and alkaline soils. The acid phosphatases show ideal catalytic activity in acidic pH, in contrast to acid phosphatases, alkaline phosphatases show their ideal catalytic activity at neutral pH value. Soil microorganisms are the main sources of phosphatase enzymes in soil (Garcia et al., 1992; Xu and Johnson, 1995). Specifically, rhizospheric soil has high phosphatase activity as compared to other soils (Tarafdar and Junk, 1987). The significant amount of phosphorous added to the soil will quickly fixed into insoluble inorganic and organic form make bonds with soil aggregates and become inaccessible to the plant system (Sanyal and De Datta, 1991).

Microorganisms especially present in rhizospheric soil release phytases and phosphatases in soil, help in mineralization of insoluble form of P into soluble form, make it accessible to the plants (Raghothama, 1999). *Aspergillus* and *Penicillium* are well reported fungi for phosphatases and phytase enzyme production (Aseri et al., 2009). Relwani et al. (2008) reported that *Aspergillus* fungi produce acid phosphatase and phytase enzyme in culture supernatant under laboratory conditions. Extracellular phytases enzyme released by microorganisms has more predominant action as compared extracellular phosphatases (Aseri et al., 2009). Decrease in soil pH makes the phytases enzymes less effective hence phytases are more active in alkaline conditions (Richardson et al., 2005). Pandey et al. (2008) demonstrated that acid phosphatase action is higher as compared to alkaline phosphatase in all the treatments. Phytic acid represent an extensive part of the organic phosphorous, still have being just ineffectively used by plants (Richardson et al., 2000). Phytases have been reported in bacteria, yeasts and fungi (Shieh and Ware, 1968).

According to the vast differences in structure, substrate specificity, pH-optimum and mechanism of hydrolysis phytases are ordered into different families. A portion of the histidine and acid phosphatases (HAPs) have wide substrate specificity and low pH-optimum on the other hand most common alkaline beta propeller phytases (BPPs) are substrate specific to just phytate particle and its complexes. The main source of phytase enzyme is oilseeds, nuts, legumes and cereal pollen grains. Phytase activity is abundant in Cereal seeds for example rye, triticale, barley, wheat and pseudo-cereal fruits like amaranth, buckwheat. Grain seeds have ten time higher phytase activity as compared to the Legumes and oilseeds (Konietzny, 2002). Maximum phytases in plant seeds are available in aleurone layer in grains and in cotyledons of legumes and huge amounts of phytate is also additionally found there (Hegeman, 2001; Konietzny, 2002). It has been observed that phytase level will increase in seed germination phase of plant life cycle that is helpful in fast

growth of seedlings (Greiner, 2007). Most plant phytases worked at temperature 55–60 °C with acidic pH-optimum averaging around pH 5.0. A maize phytase showed its extreme activity at 55 °C temperature and pH of 4.5, in case of soybean phytase GmPhy showed optimum pH 4.5–5.0 and is steady up to 60 °C temperature (Hegeman, 2001). Some of the phytases found in roots are have low hydrolytic activity and are not release into rhizosphere. Phytase enzyme produced by *Arabidopsis thaliana* roots are less than 0.8% of total root acid phosphatase activity (Richardson et al., 2001a). Because of this, phytase will not able to give off an impression of being an extracellular enzyme. In this way, *A. thaliana* and different plants are for the most part unfit to develop on agar medium has phytic acid as the main source of phosphorus (Richardson et al., 2001a). Different treatments with wheat in research center conditions gave results that low phytase activity in plant roots is the main reason that constraining wheat capacity to acquire P from phytate (Richardson et al., 2000). As compared to the plant root system number of soil microbes is known to produce phytase enzymes. Nowadays fungi, yeast and some members of *Bacillus* and *Enterobacter* are known to produce probably the most dynamic extracellular phytases. Phytases produced by *Bacillus* and *Enterobacter* genera have high substrate specific activity, pH-optimum in range of pH 3.5–7.5 and temperature optimum at 37 °C to 70 °C (Lei and Stahl, 2001; Gontia et al., 2012). Some of the alkaline *Bacillus* phytases are restricted only to phosphomonoester bonds in phytate molecules due to their narrow substrate specificity (Oh et al., 2004).

15.10 Molecular approach of microbial phytases as biofertilizers

High agricultural efficiency later on will to a great extent rely upon the proceeded with innovative technique to decrease synthetic chemical fertilizer dose and the expense of crop production. Utilization of microbial phytases is in this manner imagined as a successful way to enhance crop yield. As plant root system is not able to hydrolyze soil phytate into simple phosphorus from, to overcome this problem plant may be treated with some bacteria responsible for secretion of phytases enzymes in rhizosphere such type of bioinoculants are known as biofertilizers. Wheat seed inoculation with soil bacterium *Pseudomonas* sp. CCAR59 having extracellular phytase activity improves the accessibility of phytate-derived phosphates to the root system (Richardson et al., 2000). *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Azospirillum* and *Burkholderia* are other well known bacterial biofertilizers, similarly *Enterobacter*, and *Pantoea* isolates are additionally able to release inorganic phosphate from phytate (Jorquera et al., 2008; Singh et al., 2014). *Enterobacter* strains isolated from the rhizospheric soil of vegetables and a strain of *Pseudomonas* sp. from Australian farming soils are also known to produce extracellular phytases and positively affect phosphorus accessibility to plant system and improve plant development (Yoon et al., 1996; Richardson, 1997). Various microorganisms isolated from *Lupinus usulbus* rhizosphere in Japan have phytate-hydrolyzing activity that can improve plant phosphorus nutrition (Unno et al., 2005). A large number and variety of microorganisms that are responsible for extracellular phytases production are inhabited in rhizospheric soil of various plants. India, China, Iran and some other countries widely use bacterial biofertilizers to improve the crop yield and soil fertility. Well known and most effective biofertilizers are *Rhizobium*, *Azotobacter*/*Azospirillum* and Phosphobacteria-based biofertilizers (Akbari et al., 2011). These biofertilizers are commonly delivered to field in granular, powder and liquid formulations (Li and Zhang, 2001). Microbial based biofertilizers are exceedingly productive option in contrast to synthetic chemical fertilizers due to their simple use, eco-friendly nature, and cost adequacy (Mazid and Khan, 2014).

Biofertilizers increase the availability of limiting nutrients, for example, nitrogen, phosphorus, group B vitamins and amino acids to the plant system and showed constructive effects on plant growth and development. In addition to the plant growth and development bio fertilizers show some additional effects like suppression of diseases caused by plant pathogens, production of plant growth regulators and reduction of ethylene levels in root cells (Idriss et al., 2002). Due to the our insufficient knowledge of the ecological, molecular and physiological role of microbial communities on plant growth farmer are lacking in widespread use of microbes as biofertilizers (Bloemberg and Lugtenberg, 2001). The most recent logical discoveries prove that phytases of microbial origin assume a major job in soil phosphorus cycle. Phytases of microbial origin are become an appealing target for industrial use, due to their possibly considerable agronomic and natural value for plant development during periods of long- term phosphorus deficiency (Jorquera et al., 2008). At last, phytases of microbial origin positively affect plant nutrition by making accessible important soil microelements typically chelated by phytate to the plant system. Hence, biofertilizers applications as in the form of liquid culture, refined microbial phytases or live phytase-delivering microbial culture can be seen as a proficient, naturally inviting way to deal with increment bioavailability of soil phosphorus and decrease the as of now boundless utilization of inorganic phosphate fertilizers.

15.11 Conclusion

In agricultural farming system phosphorous is provided to crop by supply of manufactured phosphatic chemicals, the use of chemical phosphate fertilizers no doubt is a costly affair to farmers and environmentally problematic. On the other hand application of phosphate solubilizing microbes as bio-fertilizers is best choice in place of chemical P-fertilizers. The applications of phosphate solubilizing bacteria also fulfill the urgent requirement of phosphate fertilizers in agricultural farming system. Depletion in rock phosphate reserve and unavailability of phosphorus from organic sources is a major area of concern across the world not only in undeveloped but also in developed countries. Therefore exploring microbes for P availability is a feasible and the use of phytases enzymes of microbial origin is increasing for potential biotechnological applications such as agriculture, environmental protection and biofertilizers. Phytases are also crucial for recycling phosphorus in ecosystem particularly from its organic form i.e. phytic acid and thus decreasing the demands of chemical fertilizers to meet phosphorus deficiency. Optimum conditions has been created by various scientists for effective phosphorus sustenance and expanded biological yield, various investigations are needed to design the best procedures and need to create propelled biotechnologies that should totally based on plant and microbial delivered phosphatases. In addition better mechanism need to be find out to understand the relationship between phytase, phytate, soil and plant physiology.

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PGPR secondary metabolites: an active syrup for improvement of plant health

Jai Singh Patel¹, Sudheer Kumar Yadav², Raina Bajpai³, Basavaraj Teli³ and Md. Mahtab Rashid³

¹Dalhousie University, Department of Plant food and Environmental Sciences, Nova Scotia, Canada., ²Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, India., ³Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India.

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16.1 Introduction

The essence of life is essentially based on the biomolecules which include nucleic acids, proteins and lipids. These are the primary metabolites which are synthesized after primary metabolism. As the organisms start reaching its maturity with the passing time, they also commence the execution of various supplementary metabolic pathways called the secondary metabolism which leads to the synthesis of secondary metabolites (Fig. 16.1). These secondary compounds are not the elemental part of normal life processes and also they are synthesized in very minute amplitudes in comparison to the primary metabolites. Many times these metabolites take part in defense reactions to oppose microorganisms and/or insect pests and some of those are produced to reverberate the pathogen attack (Mehnaz, 2013). The secondary metabolites are produced in bacteria at its stationary phase of growth. Almost all of these metabolites are secluded into the medium of growth and are readily derivable. Upon the change in the conditions of growth and the compositions of media, there is a decrease or increase in secondary metabolites' production. Pseudomonads among the bacteria are well distinguished for producing secondary metabolites (Table 16.1). These metabolites have a major part to play in the producer's defense actions as well as it also renders support to the plants they are mingled with. Amidst the Pseudomonads, secondary metabolic products of fluorescent *Pseudomonas* spp. are examined properly. The metabolites' isolation and identification, as well as the genes engaged in their biosynthesis, have been well described. *P. aeruginosa*, *Pseudomonas fluorescens*, *P. aureofaciens*, *P. pyrocinia* and *P. putida* are some of the species of fluorescent *Pseudomonads* which have exhibited to possess antifungal action with a vacillating extent of antagonism (de Weger et al., 1986). The antimicrobial action of pseudomonas is a repercussion due to the production of several classes of metabolites such as 2-4-diacetyl phloroglucinol, cyclic lipopeptides (CLPs), phenazines, pyoluteorin, rhizoxin and pyrrolnitrin (Liu et al., 2007; Loper et al., 2008).

The phytopathogens are the reason behind the plant diseases which in turn leads to the considerable loss in crop production. Due to the environmental concerns posed by the chemical pesticides in recent times, there is an increase in the search of biological control of the plant disease as a putative alternative (Patel et al., 2016; Patel et al., 2017). Synthetic pesticides not only degrade the environment but also are costly and most importantly they are harmful to humans and

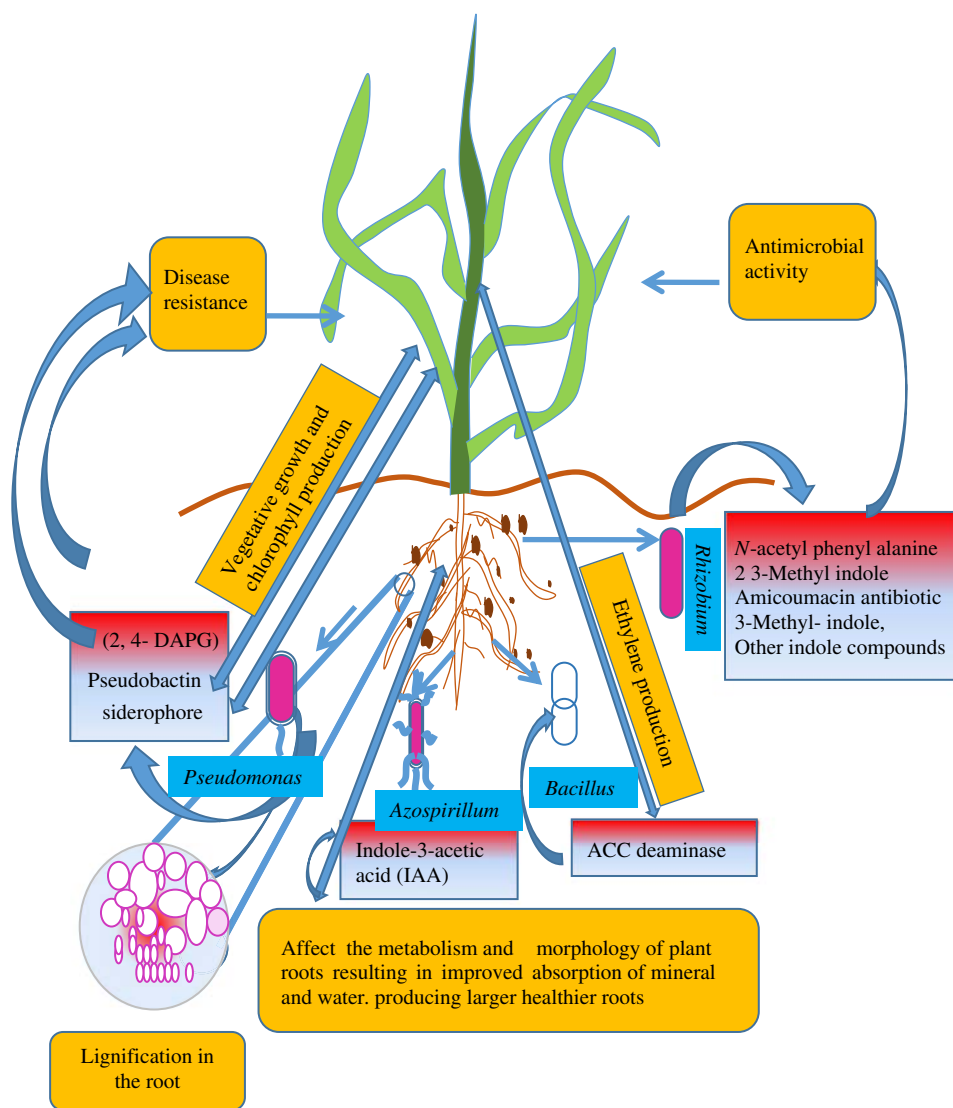


FIGURE 16.1 Secondary metabolites production and their mode of action by PGPR.

animals. In addition to the above-stated problems posed by the synthetic pesticides, the indiscriminate and repeated application of them for disease control, encourage the evolution of resistant pathogen strains. Beneficial microbes are known to produce certain molecules which are biologically active and affect the plant-pathogen interactions (Vinale et al., 2014; Srivastava et al., 2014; Kumar et al., 2017). The recent inclination of agricultural practices towards organic farming has brought the use of microorganisms into the limelight which is analogous to synthetic fertilizers and pesticides (Keswani et al., 2014). Some of these beneficial microbes produce many secondary metabolites possessing antibiotics, which enables them to restrict and/or execute other microorganisms to reduce its competition for nutritional niche (Vinale et al., 2014). Plant growth-promoting rhizobacteria (PGPR) is a disparate faction of microorganisms that are known to assist in the plant's primary productivity through growth promotion and for which they are progressively comprehended (Sarkar et al., 2014). PGPR also functions in a consortium mode leading to an embellished yield and nutrient content in the plants (Patel et al., 2016, 2017). The consortium of beneficial microbes leads to an increment in the antioxidants level of crop plants spatially in the edible parts, consumption of which could be an efficient method to combat the oxidative stress in humans and livestock (Yadav et al., 2013; 2017). Furthermore, PGPR also incites the plant's defense response by induced systemic resistance (ISR), through which it makes an imperative augmentation in the biological control of pests and phytopathogens (Kartikay et al., 2016). The efficacy of PGPR-incited plant defense is although depends on an array of genetic and abiotic/biotic environmental elements. In natural condition, PGPR is known to be present within the complex neighborhood of soil microorganisms that are an inhabitant of root rhizosphere

TABLE 16.1 List of secondary metabolites produced by PGPR and their various mechanism of action.

Sl. No.	Secondary metabolite(s)	Organism(s)	Plant(s)	Function(s)	Reference(s)
1.	<i>N</i> -acetyl phenylalanine, 2 3-methyl indole, amicoumacin antibiotic, 3-methyl- indole, Indole compounds	<i>Rhizobium leguminosarum</i> LNRLS9	<i>Dolichos lablab</i>	Antimicrobial activity against <i>Bacillus subtilis</i> , <i>Escherichia coli</i> , <i>Aspergillus niger</i>	Kumar et al., 2017
2	2, 4- Diacetylphloroglucinol (2, 4- DAPG)	<i>Pseudomonas</i> spp.	<i>Triticum aestivum</i>	Acts against <i>Gaeumanomyces graminis</i> var. <i>tritici</i>	de Souza et al., 2003
3.	Pseudobactin siderophore	<i>Pseudomonas putida</i> B10 strain	<i>Beta vulgaris</i>	Suppress soil borne fungi <i>Fusarium oxysporum</i> in iron deficient soils	Kloepper et al., 2004
4.	2,3-Butanediol and acetoin (Volatile organic compounds)	<i>Bacillus subtilis</i> GB03, <i>Bacillus amyloliquefaciens</i> IN937a	<i>Arabidopsis thaliana</i>	Induction of ISR against <i>Erwinia carotovora</i>	Ryu et al., 2003
5.	Phenazine-1-carboxamide	<i>Pseudomonas chlororaphis</i> PCL1391	<i>Solanum lycopersicum</i>	Act against <i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	Chin-A-Woeng et al., 2001
6.	2-Hexyl 5-propyl resorcinol	<i>Pseudomonas fluorescens</i> PCL1606	<i>Persea americana</i>	Biocontrol for <i>Dematophora</i> root rot	Cazorla et al., 2006
7.	2,4-Diacetylphloroglucinol and extracellular proteolytic activity	<i>Pseudomonas fluorescens</i> F113 and <i>Stenotrophomonas maltophilia</i> W81	<i>Beta vulgaris</i>	Biocontrol for <i>Pythium</i> sp. causing damping-off	Dunne et al., 1998
8.	Viscosinamide	<i>Pseudomonas</i> spp. strain DR54,	<i>Beta vulgaris</i>	Affect sporulation of <i>Pythium</i> zoospores. Affect mycelia of <i>Rhizoctonia solani</i>	Thrane et al., 2000

Abiotic stress

1.	Uptake of the Fe-pyoverdin complex	<i>Pseudomonas fluorescens</i>	<i>Arabidopsis thaliana</i>	Increase iron level in plant tissue and improved plant growth	Vansuyt et al., 2007
2.	Siderophore and Fe-regulated compounds	<i>Pseudomonas fluorescens</i> WCS 374	<i>Raphanus raphanistrum</i>	In response to <i>Fusarium</i> sp	Leeman et al., 1995
3.	Indole-3-acetic acid (IAA)	<i>Azospirillum</i> spp.	<i>Oryzae sativa</i>	Alters physiology and metabolism of rice roots with better absorption of mineral and water	Bashan et al., 2004; Perrig et al., 2007
4.	Indole-3-acetic acid (IAA)	<i>Bacillus subtilis</i> AK31, <i>Pseudomonas fluorescens</i> AK32 and <i>Bacillus</i> spp. AK21.	<i>Triticum aestivum</i>	PGPR inoculated seeds showed a good level of IAA production at different time intervals	Karnwal, 2012
5.	Auxin signaling in plants (IAA)	<i>Phyllobacterium brassicacearum</i> STM196	<i>Arabidopsis thaliana</i>	Soil inoculation designed the typical root architecture	Contesto et al., 2010

(Continued)

TABLE 16.1 (Continued)

Sl. No.	Secondary metabolite(s)	Organism(s)	Plant(s)	Function(s)	Reference(s)
6.	Auxin regulation	<i>Pseudomonas fluorescens</i> WCS365	<i>Raphanus raphanistrum</i>	A significant increase plant growth of radish	Kamilova et al., 2005
7.	Glucose and abscisic acid sensing	<i>Bacillus subtilis</i> GB03	<i>Arabidopsis thaliana</i>	In response to stress	Zhang et al., 2008
8.	Auxin homeostasis	<i>Bacillus subtilis</i> GB03	<i>Arabidopsis thaliana</i>		Zhang et al., 2007
9.	ACC deaminase	<i>Achromobacter piechaudii</i> ARV8	<i>Solanum lycopersicum</i>	Decrease in the level of ethylene. Prevention of the inhibition of plant growth in tomato plants grown under salinity stress	Mayak et al., 2004
10.	Auxin	<i>Bacillus</i> spp.	<i>Solanum tuberosum</i>	Seedling dip	Ahmed and Hasnain, 2010
11.	Cytokinin synthesis	<i>Bacillus</i> spp.	<i>Cucumis sativus</i>	Seedling dip	Sokolova et al., 2011
12.	Gibberellins synthesis	<i>Bacillus</i> spp.	<i>Capsicum</i> spp.	-	Joo et al., 2005
13.	Absciscic acid (ABA)	<i>Azospirillum brasilense</i> Sp245	<i>Arabidopsis thaliana</i>	The increase of ABA content in the plant grown under osmotic stress	Cohen et al., 2008.
14.	Lignin accumulation	<i>Pseudomonas fluorescens</i> 63-28 R	<i>Pisum sativum</i>	Induced deposition of lignin in root cells. Inhibited colonization of the <i>Pythium ultimum</i>	Benhamou et al., 1996
15.	Polygalacturonase	<i>Azospirillum irakense</i>	<i>Oryzae sativa</i>	Induced in root cells	Sekar et al., 2000
16.	Absciscic acid (ABA)	<i>Bacillus megaterium</i> BOFC15	<i>Arabidopsis thaliana</i>	Inoculated plants exhibited higher drought tolerance created artificially by PEG	Zhou et al., 2016
17.	Pyrroloquinoline quinone	<i>Pseudomonas fluorescens</i> B16	<i>Solanum lycopersicum</i>	Plant Growth Promotion	Choi et al., 2008
18.	ACC deaminase	<i>Pseudomonas putida</i> UW4		Plant growth promotion	
19.	VOC - polyamine, spermidine,	<i>Bacillus megaterium</i> BOFC15	<i>Arabidopsis thaliana</i>	Induces polyamine production and increase plant biomass, root structure and photosynthesis	Zhou et al., 2016
Nutrient uptake/biofertilizer					
1.	Nitrogen uptake	<i>Serratia proteamaculans</i> or <i>Serratia liquefaciens</i> . <i>Bradyrhizobium japonicum</i>	<i>Glycine max</i>	Increased fixation of nitrogen. Increased number of root nodules and the plant biomass, at 25 °C as well as at 15°C	Bai et al., 2002
2.	Potassium solubilization	<i>Bacillus</i> spp.	<i>Cucumis sativus</i> , <i>Piper nigrum</i>	Seedling inoculation	Han and Lee, 2005

(Continued)

TABLE 16.1 (Continued)

Sl. No.	Secondary metabolite(s)	Organism(s)	Plant(s)	Function(s)	Reference(s)
3.	Biological N fixation	<i>Rhizobium</i> isolates LAR-10 and LAR-14,	<i>Arachis hypogaea</i>	N Uptake and Salt tolerance	El-Akhal et al., 2013
4.	Uptake of N and K	<i>Achromobacter</i> spp. strain U80417	<i>Brassica napus</i>	The increase of both NO^{-3} and K^{+} net influx rates	Bertrand et al., 2000
Biotic elicitor					
1.	Ajmalicine, serpentine	<i>Pseudomonas fluorescens</i>	<i>Catharanthus roseus</i>	Seedling treatment by soil drenching	Jaleel et al., 2007; 2009
2.	Hyoscyamine and scopolamine	<i>Pseudomonas putida</i> and <i>Pseudomonas fluorescens</i>	<i>Hyoscyamus niger</i>	Seedling radicle and culture media for <i>in vitro</i> growth	Ghorbanpour et al., 2010
3.	Picrocrocin, crocetin and safranal compounds	<i>Bacillus subtilis</i>	<i>Crocus sativus</i>	Soaking corms and soil drenching	Eldin et al., 2008
4.	Tanshinone	<i>Bacillus cereus</i>	<i>Salvia miltiorrhiza</i>	Hairy root culture	Zhao et al., 2010

(Sarma et al., 2015). Thus, making the understanding about the biotic interactions of PGPR with these rhizospheric microbes important in order to judge the capability of PGPR to enhance defense response in plants (Hol et al., 2013). As per Sarkar et al. (2014), S1 and Cgr strains of *Pseudomonas* were found to incite more activity of PPO (Poly Phenol Oxidase) in the consortial mode and also enhanced the activity of PPO in chickpea when infected with *Sclerotinia sclerotiorum* and under salt stress. Likewise, *Pseudomonas aeruginosa* PW09 (wheat endophytic bacterium) relieve the stress caused by *Sclerotium rolfii* (biotic stress) and 150 mM NaCl (abiotic stress) in cucumber plant (Pandey et al., 2012). A decisive role have been played by plant growth promoting rhizobacteria (PGPR) and its metabolic products in control of chickpea wilt disease caused by phytopathogen *Fusarium oxysporum* f. sp. *Ciceris* (FOC) and plant growth promotion under field and greenhouse conditions (Srivastava et al., 2014). A very early process of lignification was incited by the strains of *Trichoderma* in chickpea under FOC challenge thus providing improved protection to chickpea plants. The secondary cell wall's lignification plays a consequential role in plants' defense mechanisms against the invading phytopathogens (Meshram et al., 2019). The secondary metabolites are produced in a greater amount by *Bacillus megaterium* than *Bacillus subtilis*. The mycelial growth of target pathogen was inhibited to a range of 26.07%–48.82% by the cell cultures; culture filtrates; and metabolic precipitates obtained with acetone, ethanol, and ammonium sulfate, under the laboratory condition. The mycelial growth of the target pathogen was inhibited more by *Bacillus megaterium* in comparison to *Bacillus subtilis*. Besides, the suppression of mycelial growth of phytopathogen was more by the metabolic precipitates than the cell cultures or culture filtrates with ethanol precipitate having the highest inhibitive effect (Abdel-Monaim, 2016). These inhibitive actions of the endophytic bacterium are due to various mechanisms which include antibiotics production, siderophore production, hydrogen cyanide production, induced resistance in the host plant, competition for space and nutrition, pathogen's enzyme inactivation, and improved development of root and plant growth (Karimi et al., 2012). The proliferation of fungus and other phytopathogens is prevented by PGPR through the production of siderophores which binds the Iron (III) present in the proximity of plant root whereby impeding the pathogen from growing near the root. This process is completed by PGPR out-sparring the phytopathogens for accessible iron. However, the plants do not get affected by this territorial soil iron depletion as most of them can withstand much lower concentrations of iron (~1000 fold less), compared to the microorganisms. Hydrogen cyanide (HCN), a secondary metabolite which is produced by the Gram-negative bacteria along with carbon dioxide (CO_2) from glycine by the catalytic action of HCN synthase. The strains of PGPR which produce HCN are able to suppress diseases but the mutant strains which are unable to do so, lose their capability of protecting plants from diseases.

Apart from this, production of many antifungal metabolites is also found which are shown of having potentiality under *in-vitro* conditions and in addition are also presumed of having the same under *in-vivo* conditions as well.

These metabolites having antifungal activity entail 2-4-diacetyl-phloroglucinol, ammonia, butyrolactones, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid (PCA), zwittermycin A, pyoluterin (Plt), pyrrolnitrin (Pln), xanthobaccin, and viscosinamide (Whipps, 2001). Even though these metabolites are effective when applied in the single form, particular combinations of these metabolites are shown to be working against certain fungi. Indole acetic acid (IAA) is produced as secondary metabolites by the microorganisms which are isolated from different crops' rhizosphere in presence of an abundant reserve of substrates. The production of longer roots is aided by indole acetic acid (IAA) through an increase in the number of root-hairs and secondary roots thus improving the uptake of nutrients by plants (Datta and Basu, 2000). Cell elongation is also stimulated by IAA via modifications of certain conditions which includes an increased osmotic constitution of the cell, cell wall synthesis, and permeability of water into the cells; decreased in wall pressure of the cells; and by activating the synthesis of some specific RNA and protein. Moreover, the hydrolytic enzymes including chitinase and glucanase produced by PGPR have a multifarious role in a diverse spectrum of distinctive biological systems. These hydrolytic enzymes are produced extracellularly and have low molecular weight with high stability. On top of that, they are also produced in multiple forms or isozymes that have a different charge, regulation, size, stability, and cell wall degradation ability (Ashwini and Srividya, 2014).

The synergism between PGPR and the plants is an intricate and complementary process. The establishment of the relation between the two starts with the release of certain compounds such as malic acid, to attract the rhizobacteria thus leading to consequential attraction of the microorganisms in the region of the rhizosphere, movement of microorganisms towards the roots, and finally their colonization (Rudrappa et al., 2008). The chemotaxis and motility of *Bacillus amyloliquefaciens* FZB42 are due to the contribution of maize root exudates (Fan et al., 2012). It is also suggested by another study that the selective signaling and recruitment of beneficial rhizobacterium *Bacillus subtilis* FB17 in *Arabidopsis* plant is through the secretion of L-malic acid from the roots of the plant (Rudrappa et al., 2008). The synthesis of bio-films is crucial for the colonization of bacteria in plant rhizosphere (Ramey et al., 2004). Subsequently, after a successful colonization process, the amino acids and carbohydrate released by the plants are taken up and utilized by the PGPR and in return, they produce many substances which are biologically active and causes plant growth promotion and/or plant disease suppression. As for the example, *Bacillus amyloliquefaciens* FZB45 produces phytases which solubilize phosphate from the organically bound phosphates leading to growth facilitation in maize seedlings (Idriss et al., 2002). Apart from the biofertilizer activity of the *Bacillus* spp., biosynthesis of certain plant hormones such as cytokinin (Ortíz-Castro et al., 2008), indole-3-acetic acid (Idris et al., 2007), and gibberellic acids (Gutiérrez-Mañero et al., 2001) by them also make up to their fame of phytostimulation activity. In recent years, there are also reports of production of the various volatile compounds by the *Bacillus* spp. that are regarded to aid in increased growth of the plant. Furthermore, *Bacillus* spp. also, produce an array of biologically active metabolites having antibiotic properties like lipopeptides and polyketides. The antagonistic effect of *Bacillus subtilis* against *Podosphaera fusca* is due to the fengycin and iturin families of lipopeptides (Romero et al., 2007). Moreover, the surfactin and fengycin kind lipopeptides also function as a bacterial impetus to incite immune responses in plant cells by the activation of induced systemic resistance (Ongena et al., 2007). The effective control of fire blight disease by plant-associated *Bacillus amyloliquefaciens* is due to the production of difficidin and bacilysin families of polyketides (Chen et al., 2009). The polyamines such as cadaverine, putrescine, spermine, and spermidine are the low molecular weight and naturally occurring polycationic compounds having positive charges which are found in bacteria, fungi, plants, and mammals. Many important physiological roles are played by the polyamines in an organism and are associated with different processes such as nucleic acid replication, transcription, translation, cell division and differentiation, and stability of membrane (Wortham et al., 2007; Kusano et al., 2008) of the polyamine, spermidine has captivated the limelight of the scientific community as it is elemental for the viability of eukaryotic cells and also due to its correlation to lateral root development (Xie et al., 2014). Present chapter describes the role of secondary metabolites produced by the microorganisms for improvement of plant health, quality, and productivity. We have also focused on the mechanisms involved for such activity and list of beneficial microorganisms, which could be used in the sustainable agriculture.

16.2 Role in plant growth promotion

Growth promotion is the well-known activity of beneficial microorganisms. Several mechanisms are involved for such activity includes either direct or indirect activity of bioagents. Production of phytohormones, antimicrobial compounds, and induction of plant defense pathways (Induced systemic resistance, Systemic acquired resistance) are major mechanisms.

16.2.1 Mechanisms

Several mechanisms that directly or indirectly promote the growth of plants are possessed by *Pseudomonas aurantiaca*. *Pseudomonas aurantiaca* does not fix the atmospheric nitrogen but all the strains that were tested for production of auxins showed production of IAA. SR1 and PB-St2 strains showed phosphate solubilization activity. PB-St2 strain also showed production of 1-Amino, cyclopropane- 1- carboxylate (ACC) deaminase enzyme. The SR1 strain of *Pseudomonas aurantiaca* has particularly been studied for its activity as a growth promoter in various crops. The colonizing strength of this particular strain was studied in soybean, wheat, and alfalfa, before starting the long-term inoculation experiments. In this strain, the CFU count was in the level of 10^5 CFU per seed for these crop plants (Andrés et al., 2011). The endophytic behavior of SR1 is also studied for different crops (Rosas et al., 2009; Carlier et al., 2008).

16.2.1.1 IAA production

The estimation of IAA production in SR1 showed that the maximum production ($11.7 \mu\text{g/mL}$) in a 24-hour old culture which decreased later on. The quantification of IAA production in PB-St2 was done by HPLC 1-week after the growth showed a very low amount ($0.15 \mu\text{g/mL}$) which may be due to its degradation as the estimation was done after one week. In the plant growth promotion, IAA is considered as a major mechanism after nitrogen fixation. Both the endogenous IAA and those produced by root/rhizosphere-colonizing microbes are believed to work in conjugation leading to stimulation of cell elongation and/or proliferation and enhanced nutrients and minerals uptake from the earth by the plants (Suzuki et al., 2003, Patten and Glick 2002). The IAA produced by the bacterium affects the growth of plants that are inoculated with the IAA-producing bacteria. Therefore, the plant growth is facilitated by the bacteria by modifying the phytohormonal balance of the treated plants (Vessey, 2003).

16.2.1.2 Phosphate solubilization

The plant's growth is limited due to the low availability of soluble phosphate ions. The process of solubilization of phosphates bounded with organic and/or inorganic compounds is performed by some bacteria and thus helping the growth of the plant. Many strains of bacterial genus *Pseudomonas* could carry out the solubilization of phosphates from the insoluble inorganic/mineral phosphate compounds like dicalcium phosphate, rock phosphate, and tricalcium phosphate (Rodriguez et al., 2006). The soluble phosphorous is released into the soil form the organic compounds by the action of different enzymes viz: phytases, phosphatases, carbon-phosphorous (C-P) lyases, and phosphonates. The enzyme C-P lyases catalyze the cleaving of carbon-phosphorous links in the organophosphonates compounds. The phosphorous release from inorganic phosphate compounds is related to organic acids production, namely gluconic acid (Rodriguez et al., 2006). The phosphate solubilization is carried on a moderate level by *Pseudomonas aurantiaca* SR1 (Rovera et al., 2008). Such enzymatic action was not shown in the PB-St2 strain or in any other *Pseudomonas aurantiaca* strains.

16.2.1.3 ACC deaminase production

The production of ACC deaminase enzyme is seen in PB-St2 strain. Although this strain hasn't been used in any plant related experiment, it is a good contender for being a bio-fertilizer due to the presence of this enzyme and apart from the production of IAA. A decrease in the level of host plant ethylene level is brought by ACC deaminase producing bacteria thus easing growth and development of the plant by catalyzing the hydrolysis of ACC (precursor of ethylene). Ammonia and α -ketobutyrate are the two products of this catalytic process which are utilized by the bacterium as a carbon and nitrogen source for improvement of plant health (Klee et al., 1991). By this technique, the bacterium lowers the level of ethylene in plants as it acts as a sink for ACC and thus, precluding the conceivably detrimental emanations which may arise due to high concentrations of ethylene (Saleem et al., 2007). The results given by ACC deaminase trait containing bacteria is highly consistent in enhancing the growth and yield of the plant and so is a potential contender for biofertilizer formulations (Shaharoon et al., 2006). The ACC deaminase producing bacteria provide relief to plants from various forms of biotic stress like effects of disease-causing bacteria, heavy metals, polyaromatic hydrocarbons, drought, and salinity (Glick et al., 2007).

16.2.1.4 Plant health improvement by *Pseudomonas aurantiaca* SR1

The promotion in the growth of the crop plants has been reported that had been inoculated by *Pseudomonas aurantiaca* SR1. In an experiment by Andrés et al. (2011), soybean and alfalfa plants were inoculated with *Pseudomonas aurantiaca* SR1 and also in a consortium either with *Sinorhizobium meliloti* or *Bradyrhizobium japonicum*. In the experiment, it

was seen that the dry weight of shoot, root, and nodule along with the length of root and shoot of alfalfa plants was increased by SR1 when inoculated in a mixture with *Sinorhizobium meliloti* 3Doh13 in comparison to those plants which were inoculated only with *Sinorhizobium meliloti* 3Doh13. Likewise, the dry weight of root and shoot along with the number of nodules increased in the soybean plants which were inoculated with SR1 in a consortium with *Bradyrhizobium japonicum* E109 in comparison to those which were inoculated only with *Bradyrhizobium japonicum* E109. Under the greenhouse conditions, it was observed that *Pseudomonas aurantiaca* SR1 formulation, led to the promotion of root development in sugarcane, wheat, and carob tree along with higher nodules count and development of root in alfalfa and soybean when co-inoculated (Rosas et al., 2005; Rovera et al., 2008).

For evaluating the role of *Pseudomonas aurantiaca* SR1 on growth promotion of crop plants under the field conditions. The strain SR1 was applied to wheat and maize seeds as seed treatment, which improves plant growth, and decreases the use of nitrogen and other chemical fertilizers. In both the crops, colonization of SR1 in the roots was seen which was insistent at pertinent population densities and a higher level of yield was produced by them under the low fertilization doses in comparison to those grown under conventional doses of fertilizers. The promotion in the growth of the plants inoculated with SR1 may be implicated due to the two main direct mechanisms viz: production of IAA and solubilization of phosphates.

16.3 Biological control

Biological control activity regarded as the most common indirect mechanism of these bacteria in promoting the growth of the plant. Fungal phytopathogens are suppressed by fluorescent Pseudomonads through the production of metabolites which are antifungal in nature and release of siderophores which are the iron-chelating agents that leads to iron sequestration in the rhizospheric region and in turn, makes the iron unavailable to other organisms (Dwivedi and Johri, 2003). The antimicrobial compounds produced by these bacteria include chitinase enzyme, cyclic lipopeptides, HCN, phenazines, and various other compound showing antibacterial and antifungal properties against the phytopathogens. Not only the population density of these bacteria is increased by the process of root colonization, but it is also a mechanism for the deliverance of secondary metabolites. There are mainly two mechanisms that are involved through which these bacteria acts as a biocontrol agent: (i) phytopathogen inhibition through the action of the secondary metabolites produced by them and/or (ii) induction of systemic resistance in the host plants. However, it is important for these bacteria (possessing biocontrol activity) in both the cases to capable of competing against other rhizospheric bacteria, establishing itself in the rhizosphere, and colonizing in roots of the host plant. If the protection of plants is through the action of secondary metabolites then a higher population density of the bacteria is required and a comparatively low density of population of the same is enough in case the mechanism of protection is by inducing the systemic resistance (Chin-A-Woeng et al., 2003).

16.4 Siderophore helps for the accumulation of nutrients

The capability of a phytopathogen to escalate in the soil is restricted by the biocontrol agents by competing against them for nutrients such as carbon, nitrogen, iron, etc (Fernando et al., 1996). The traits which are considered important under this mechanism of control are generation period of the bacterium should be short, the rate of colonization, and the extent of colonization of the bacterium in the root system of the plant. The colonization of bacteria in rhizosphere is generally restricted around smaller area of the area available for colonization and presumably conforms to the common nutrient accessible regions principally at the junctions present between epidermal cells of the roots (Chin-A-Woeng et al., 1997). The phytopathogen is considered to be outcompeted if it does not have sufficient nutrients for proper survival and reproduction.

One of the most well-established observation of competition for nutrients is a limited amount of iron works as constitutional growth cofactor for all the living beings. The bio-availability of soluble iron (III) ions is restricted to the alkaline and neutral pH for the soil microbes. Under the iron (III) limiting conditions, the fluorescent Pseudomonas are known to release siderophores from their cells to take up the iron (III) ions as they are high-affinity iron chelating agents. A siderophore-iron (III) complex is formed after the binding of siderophores to the iron (III) ions which consequently combined with the limited iron dependent receptors present on the surface of the bacteria. The ferric ion is then released in the cell cytoplasm of the bacterial cells where it gets activated as iron(II)/ferrous ions. The high-affinity siderophore-producing bacteria can cause inhibition of the fungal phytopathogens in the rhizospheric region when the concentration of ferric ions is low (Schippers et al., 1987). The mutant strains which were deficient in producing siderophores were found to be less suppressive to the phytopathogens in comparison to the isogenic parental strains (Bakker et al., 1986).

16.5 The role phenazines and compounds responsible for its production

Phenazines is a broad-spectrum antibiotic and antifungal compound which make heterocyclic nitrogen compounds family larger in antibiotic compounds. But, the evaluation of these compounds for the role of biological management is much limited because of adequate information regarding the mode of action as an antifungal agent. Mahajan et al. (1999), evaluated few phenazines to draw a valid conclusion for the mode of action indicating that phenazines diffuse or insert across the membranes and behave as reducing agent by coupling the oxidative burst reaction leading to leakage of superoxide radicles and hydrogen peroxide in the intercellular regions resulting harmfulness towards organisms.

Various factors limit the production of secondary metabolites i.e. organism internal factor and the favorable environmental conditions. Bacteria are known to secrete metabolites with their role as an antifungal agent through the population density gene expression. Quorum sensing helps in communicating among the bacterial population in the given environment condition by adjusting all depending factors. Self-inducer signal molecules provide population density news from the neighbor sister cells to the other cell. However, in Gram-negative bacteria, production of class *N*-acyl, L-homoserine lactones (*N*-AHL) is the most studied. *N*-AHL have ability for the regulation of the several compounds involved in bioluminescence effect till virulence activity and production of a secondary metabolic compounds. In-vitro culturing of *Pseudomonas* spp. leads to the production of phenazines through cell density-dependent production of these compounds. Production of phenazines in a mid-exponential growth phase, lag and early stationary and late exponential stage allows the bacterial species to produce the phenazines (Chin-A-Woeng et al., 2003). Expect growth and environment condition, gene expression can also play role in the production of such phenazines during its production. The bio-availability of certain nitrogen and carbon sources, components of major root exudates, oxygen status, and metal ions (Chin-A-Woeng et al., 2003) and growth medium (Seveno et al., 2001) can vary the production of phenazines compounds. So, growth, medium and environment conditions decide the stable production of phenazines. Therefore, this is quite necessary to understand the nutrient requirement and conditions of the environment for the growth and development of healthy bacterial colonies for the production of phenazines further, effectively utilizing them has a potential source of bioagents for the plants.

16.6 Antagonistic activity of lipopeptides (LP)

Lipopeptides (LPs) has a lipid tail attached to a short cyclic or linear oligopeptide. Different fungi and bacteria like *Streptomyces*, *Bacillus* and *Pseudomonas* produce lipopeptides. LPs have gained significant attention for its properties such as antimicrobial, antitumor, cytotoxic, suppressor of immunity, and surface sterilizers (Gross and Loper, 2009). Important way of action of LPs proposed is the formation of a pore in membranes causing disparity in transmembrane ion fluxes leading to the death of the cell (Baltz, 2009). Many LPs have been also reported for their antifungal activities. Fungal growth with enhanced branching and swollen hyphae is inhibited by LPs. Diminished actions of mitochondria and esterases, altered mitochondrial organization, reduced intracellular pH, and reduced hydrophobicity of hyphae also cause inhibition effect (Thrane et al., 1999). LPs produced by *Pseudomonas* spp. has the capacity to lyse zoospores thus showing critical influence on pathogens of oomycetes like *Pythium* and *Phytophthora* spp. LPs of the viscosin group has been well characterized for this effect (Raaijmakers et al., 2010). Nybroe and Sørensen (2004), have also reported antiviral activity of a viscosin group of LPs against enveloped viruses. For cyclic LPs formation *N*-AHL (*N*-3-acyl-hydroxyoctanoyl-HSL)-based quorum sensing was known to be involved in few plant pathogenic *Pseudomonas* strains like *Pseudomonas fluorescens* strain 5064. In the mutants, either adding up of culture extracts or the synthetic signal molecules could re-establish viscosin formation (Cui et al., 2005). *Pseudomonas putida* strain PCL1445, four *N*-AHLs were reported (Dubern et al., 2006) to be having the role in the regulation of putisolvin biosynthesis. Quorum sensing based on function of *N*-AHL was not observed in biosynthesis LP in other *Pseudomonas fluorescens* strains (Raaijmakers et al., 2010). Thus, it concludes that on changing of species and strains. There is variation in biochemical and molecular basis of QS-dependent regulation of LP biosynthesis.

16.7 Systemic resistance induced by secondary metabolites

Systemic acquired resistance (SAR) is the resistance triggered by pathogen infection (Hunt et al., 1996) linked to augmented level of salicylic acid (van Loon, 1997) followed by the pathogenesis-related (PR) proteins activation (Gaffney et al., 1994) whereas induced systematic resistance (ISR) is resistance induced by “nonpathogenic” biocontrol bacteria (van Loon et al., 1998). Ethylene and Jasmonic acid are required for ISR response (van Wees et al., 1997); though lipopolysaccharides, siderophores, or flagella, can also activate it (Leeman et al., 1995). It is supposed that ISR is due to

the closer relation between the host plant and inducing agent (van Wees et al., 1997). In tomato and bean, phenazine derivative pyocyanin was observed to participate in the induction of ISR against *Botrytis cinerea*. reported that salicylic acid or pyocyanin mutant of *Pseudomonas aeruginosa* 7NSK2 or its wild type was not able to trigger resistance against *Botrytis cinerea* (Audenaert et al., 2001). It was put forward that pyochelin and pyocyanin both actively participate in the formation of reactive oxygen species causing harm to cell and eventually inducing resistance. The pyochelin is formed by the precursor salicylic acid which is basically a siderophore (Audenaert et al., 2001). Nonpathogenic *Pseudomonas* strains induce defense responses in plants by producing LPs produced against oomycetes and pathogenic fungi. Treatment of roots of tomato plants with purified massetolide-A which is product of *Pseudomonas fluorescens*, induced resistance in leaves against *Phytophthora infestans* infection (Tran et al., 2007).

16.8 Conclusion

PGPR has been found from various region of the universe, from various sources including oil, sludge and plants. Most of the them are known for the antibiotics production and phenazines production. Several other PGPR were also known for the production of cyclic lipopeptides, HCN, siderophores, protease, pyoverdin, IAA, enzymes for solubilization of phosphate, and various other metabolites. Antimicrobial activity of PGPR observed against several important phytopathogens. Plant Growth promotion of different crops is reported by seed priming. Several industries are producing different commercial formulations of these PGPR. Different strains of *Pseudomonas* strains have already been marketed as commercial biocontrol products, and one of them is “Cedomon” (BioAgri AB, Uppsala, Sweden). Seed biopriming based on a *Pseudomonas chlororaphis* strain, providing protection against different seed-borne phytopathogens in barley plants. Such products are successfully marketed since more than 10 years in most of the European countries. “Mycotoxin” reported as antifungal bio-pesticide obtained from *Pseudomonas aurantiaca* M-518. Plant beneficial the traits of *Pseudomonas aurantiaca*, suggested that other pseudomonads such as *P. putida* and *Pseudomonas fluorescens* could be other species of *Pseudomonas* which can be used for crop protection and yield improvement. Biopesticides and biofertilizers are currently the hot are of research. The PGPR could serve as ecofriendly source for sustainable agriculture and alternatives for harmful chemicals and pesticides.

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Volatile organic compounds mediated plant-microbe interactions in soil

Waseem Raza and Qirong Shen

Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing, China

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17.1 Introduction

Rhizosphere is an intricate ecological environment around plant roots, where different kinds of microbes interact and communicate with plants and each other for their mutual benefits (Ho et al., 2017; Lugtenberg et al., 2017). Different natural inhabitant bacterial and fungi have been reported for their role in pathogen inhibition, plant growth promotion and induction in systemic resistance in plants (Bulgarelli et al., 2012; Vejan et al., 2016; Amavizca et al., 2017; Kandel et al., 2017). Several reports describe the mechanisms of action of soil microbes to promote plant growth, inhibit pathogen invasion and induction in systemic resistance in plants. For example, some microbes fix atmospheric nitrogen by establishing a symbiotic relationship with plants and several have been found to provide micronutrients (Soleimani et al., 2005; Meldau et al., 2013). Some microbes secrete siderophores and phosphatases as chelating agents to help plants in nutrient uptake, especially iron and phosphorus (Zhang et al., 2009). Similarly, some microbes produced plant growth promoting hormones (e.g., auxin, cytokinin, and gibberellins) and others produce antibiotics (e.g., iturin, fusaricidins, 2,4-diacetyl-phloroglucinol, phenazines) which inhibit pathogens directly to sustain and improve plant growth and health (Dodds and Rathjen, 2010; Denance et al., 2013). In addition, soil microbes produce a vast variety of volatile organic compounds (VOCs) that can generate drastic changes in plant growth and health (Raza et al., 2017; 2016a; Bailly and Weisskopf, 2017). VOCs are low-molecular-weight lipophilic compounds that can modulate several features of plant growth and health. There is growing evidence that VOCs can promote plant growth, suppress pathogens and induce plant defense against biotic and abiotic stresses (Kanchiswamy et al., 2015). The VOCs can diffuse through the atmosphere and porous soils, and can thus show an effect on longer distances than other microbial compounds (Leff and Fierer, 2008). These compounds offer a tremendous potential for sustainable agriculture, but to date, only a few

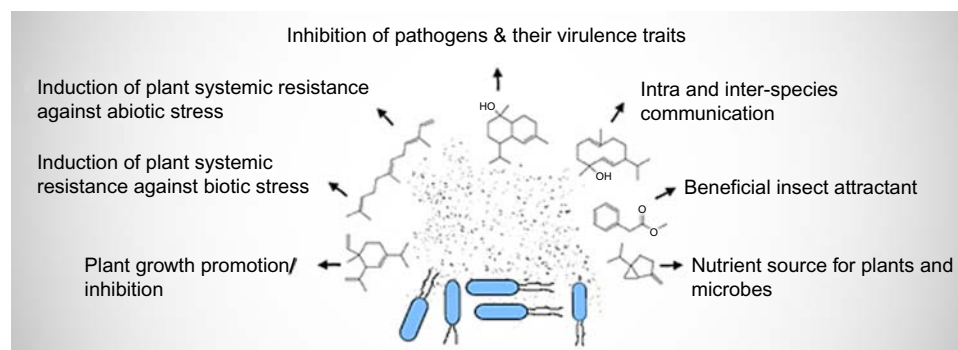


FIGURE 17.1 Different known functions of microbial volatile organic compounds.

microbial VOCs have been examined under natural conditions as their importance was only well acknowledged in the last decade (Cortes-Barco et al., 2010; Raza et al., 2015). Each microbe produces a specific mixture of VOCs that plays important roles not only in the life cycle of microbes but also in their interactions with other organisms including microbes and plants. For example, some microbial VOCs improve plant growth (Ryu et al., 2003), some suppress pathogen and hinder their virulence traits (Audrain et al., 2015), while some modulate bacterial motility, biofilm formation and antibiotic production (Kim et al., 2013). Furthermore, each microbial VOC does not essentially offer a single role. For example, a VOC indole suppresses or even kills nematodes and pathogens (Anyanful et al., 2005), mediates resistance to antibiotics (Audrain et al., 2015), increases biofilm formation in microbes (Bailly et al., 2014) and induces plant growth (Bansal et al., 2010).

Microbial VOCs are chemical language that microbes use to interact with other organisms. Hundreds of different microbial VOCs, comprising alkanes, alkenes, alcohols, esters, ketones, sulfur compounds, terpenoids, etc. have been identified; however, there is only a very limited knowledge on which VOCs can impact plant health and growth (Ryu et al., 2003), given the colossal pool of molecules that can be used in sustainable agriculture, mining in the microbial VOCs pool may offer an effective and flexible toolbox to improve plant health. Microbial VOCs can act as plant growth promoter or protector against abiotic and biotic stress while some act as both. For example, VOCs 2, 1,3-butylene glycol and 3-hydroxybutanone improved the *Arabidopsis* growth by modulating the cytokinin signaling pathway (Ryu et al., 2003). A microbial VOC dimethyl disulfide improved plant growth by enhancing the availability of reduced sulfur, and protected plants indirectly by inducing systematic resistance and directly by inhibiting pathogens *Botrytis cinerea* and *Cochliobolus heterostrophus* (Huang et al., 2012a,b). The VOCs of *Paenibacillus polymyxa* WR-2 reduced the growth of *Fusarium oxysporum* (Raza et al., 2015). While the VOCs of *Bacillus amyloliquefaciens* IN937a induced systemic resistance in *Arabidopsis* by a signaling pathway independent of ethylene, VOCs of *Bacillus subtilis* GB03 operated through a pathway that require ethylene (Ryu et al., 2004). Furthermore, the antimicrobial action of VOCs produced by *Bacillus amyloliquefaciens* strains T-5 and SQR-9 against *Ralstonia solanacearum* was reported (Raza et al., 2016b,c). There is plenty of preliminary data existing about the potential of VOCs to improve plant growth and disease suppression; however, almost all studies were conducted *in vitro*; only some studies showed that VOCs can even enhance the biocontrol effect of microbes under natural conditions (Yi et al., 2013). There is a huge pool of bioactive microbial VOCs, yet only a few VOCs-mediated plant-bacteria interactions have been extensively documented. These first results are extremely promising and the use of VOCs for sustainable crop production is the hot topic of the time. In this chapter, we will summarize different mechanisms by which microbial VOCs improve plant growth and health and VOCs secreted by plant roots affect surrounding organisms including microbes, nematodes, etc (Fig. 17.1).

17.2 Volatile organic compounds-mediated plant growth promotion

It is well known that soil microbes have potential to improve plant growth from a distance without any contact via the production of VOCs. Ryu et al. (2003) first time reported the increase in plant growth by the production of VOCs (2,3-butanediol and acetoin) by *Bacillus subtilis* GB03. In another report, a VOC 2-pentylfuran produced by *Bacillus megaterium* XTBG34 promoted plant growth by two-folds (Zou et al., 2010). Similarly, *Pseudomonas fluorescens* SS101 produced VOCs (E-11,13-Tetradecadien-1-ol, methyl ethyl ketone, and 2-methyltridec-1-ene) that enhanced the growth of *Nicotiana tabacum* (Park et al., 2015). VOCs of a soil bacterium *Pseudomonas simiae* AU increased the expression of cell division integrity maintaining proteins that improved the growth of soybean seedlings (Vaishnav et al., 2015). Detailed research depicted that VOC-mediated plant growth promotion is the result of regulation of plant hormones,

such as auxins, gibberellins, cytokinin, ethylene, etc. (Glick, 2005; Bailly and Weisskopf, 2012; Xie et al., 2009). In addition, increase in the uptake of N, P and Fe by plants was also determined after the exposure to VOCs. (Kim et al., 2011; Ortíz-Castro et al., 2014). Using microarray technique, the RNA transcripts from *Arabidopsis* seedlings after exposure to the VOCs produced by *Bacillus subtilis* GB03 showed differential expression of 600 genes related to different plant processes (Zhang et al., 2007). The VOCs not only improve plant growth, total biomass and leaf surface area but also positively influence the root development, seed germination, early flowering, no. of flowers and fruit and seed production (Zhang et al., 2007; Xie et al., 2009; Song and Ryu, 2013). Furthermore, VOCs produced by *Bacillus badius* M12 improved morphogenesis and browning of callus of tobacco (Gopinath et al., 2015). The VOCs of both bacterial and fungal were found to increase cell wall expansion and rigidity by inducing *Expansin* genes, important for the extension of cell wall (Zhang et al., 2007; Minerdi et al., 2011).

Roots are important part of plants and provide water and minerals to plants from the soil. Root exudates provide favorable environment for soil microbes by providing them nutrients, which in return provide nutrients and plant growth promoting hormones, induce systemic resistance against biotic and abiotic stress and produce VOCs that improve plant growth and roots architecture. Microbial VOCs, dimethylhexadecylamine and indole not only improved primary and lateral root length and numbers but also improved root hair density ultimately increased root surface area and volume (Bailly et al., 2014; Castulo-Rubio et al., 2015). In contrast, there are microbial VOCs that suppressed primary root formation but increased lateral roots growth and root hairs density. For example, VOCs of *Bacillus* spp. reduced primary root length and size of meristem on root tips but increased the size and number of lateral roots (Gutiérrez-Luna et al., 2010). Similarly, Meldau et al. (2013) reported that a VOC dimethyl disulfide increased lateral root formation by two folds in *Nicotiana attenuata*. Increasing the root volume would be very important for plants, which not only support a greater number of microbes around the roots, but also help plants to uptake more minerals and water. Most of the information regarding the effect of VOCs on plant growth is provided using I-plate systems and actual concentration of VOCs in the rhizosphere is also not known; however, it has been reported that plant respond better to the low concentration of VOCs (Ryu et al., 2003; Bailly et al., 2014; Bhattacharyya et al., 2015).

17.3 Volatile organic compounds-mediated modulation of plant photosynthesis

Microbial VOCs improve plant growth and health by different ways and one of those is increasing plant photosynthesis and carbohydrate accumulation. For example, Zhang et al. (2009) and Castulo-Rubio et al. (2015) reported that microbial VOCs increased photosynthesis by improving chlorophyll content in plants. This effect is mediated by two different ways. One of those is iron uptake by plants, which is vital for electron transport chain action, chlorophyll production and photosynthesis (Briat, 2007). Zhang et al. (2009) showed in *Arabidopsis* that VOCs of *Bacillus subtilis* GB03 acidify rhizosphere by increasing proton release 3-folds, thus improve iron solubility and uptake, which is then converted to Fe^{2+} and taken up by plants via iron-regulated transporter 1 (*IRT1*). In this study, the iron contents in VOC-exposed plants were double than the control treatment. In another report, Wang et al. (2017) reported that VOCs of *Bacillus amyloliquefaciens* BF06 increased the buildup of nitric oxide (NO) in *Arabidopsis*, which acts upstream of Fe-deficiency-inducing transcription factor 1 (*FIT1*) under Fe-deficiency conditions and mobilize and chelates iron.

The second mechanism is the increase of adverse response of sugar accumulation on photosynthesis by microbial VOCs. The experiments showed that *Arabidopsis* hexose sensor kinase (*HXK1*) negatively regulates photosynthesis by sensing hexose sugar concentration (Cho et al., 2010). VOCs of *Bacillus subtilis* GB03 accumulate 60% more hexose sugar in *Arabidopsis* than the control treatment; however, those VOCs also repress *HXK1* signaling (Zhang et al., 2008b). Similar kind of results were also reported by other researchers. VOCs of *Alternaria alternata* improved plant growth (~4-fold) and photosynthesis rate by accumulating higher contents of glucose, fructose, sucrose and starch and by repressing the expression of *HXK1* signaling (Sánchez-López et al., 2016).

17.4 Volatile organic compounds-mediated increase in mineral uptake by plants

It is well known that soil microbes improve plant uptake of mineral nutrients by different ways (Meldau et al., 2013; Aziz et al., 2016). The production of VOCs is one of those mechanisms by which soil microbes increase the plant uptake of mineral ions, for example, iron, copper, selenium and sulfur. Castulo-Rubio et al. (2015) reported increased uptake of iron by both mono and dicot plants using VOCs of *Arthrobacter agilis* UMCV2. Most interesting results were obtained under alkaline conditions, when the VOCs of *Bacillus subtilis* GB03 increased iron uptake by 2-fold in *Arabidopsis* (Zhang et al., 2009; Wang et al., 2017). In another report, selenium contents were 23% higher in plants exposed to VOCs of *Bacillus amyloliquefaciens* BF06. The mechanism behind was reported to be the increased

expression of sulfate transporter genes (Wang et al., 2017). The VOCs of a *Trichoderma* sp. triggered the induced systemic resistance and iron uptake in *Arabidopsis* through their effect on transcription factor MYB72 (Martinez-Medina et al., 2017). Microbial VOCs are also consumed as a nutrient source by plants, for example, *Atractylodes lancea* was reported to uptake nitrogen-containing VOCs as a nutrient source (Zhou et al., 2016). Similarly, a VOCs dimethyl disulfide was consumed by *Arabidopsis* as a source of sulfur in sulfur-deficient medium (Meldau et al., 2013). These findings clearly demonstrate that microbial VOCs not only improve nutrient uptake by affecting different genes but can also be used as a nutrient source by plants, which should be considered to design integrated nutrient management strategies.

17.5 Volatile organic compounds-mediated increase in quality and yield of plants

Microbial VOCs have also been reported to increase the quality and yield of crop plants. The VOCs produced by *Bacillus subtilis* GB03 along with a VOC benzaldehyde not only increased the growth but also the oil contents in *Atractylodes lancea* and *Codonopsis pilosula* medicinal plants (Wu et al., 2016; Zhou et al., 2016). Similarly, microbial VOCs increased sugar contents in plants where the sugar contents are considered important for the quality of crops like potato etc. (Zhang et al., 2008b; Ezquer et al., 2010; Sánchez-López et al., 2016). There are some reports that showed that microbial VOCs are also a tool to improve the flowering and fruit production in plants. For example, two VOCs 3-pentanol and 2-pentanone increased the yield by 4–6-fold under field conditions without affecting the growth of cucumber (Song and Ryu, 2013). Similarly, VOCs produced by *A. alternata* and *Bacillus subtilis* GB03 improved the time of flowering, number of silique and production of seed in *Arabidopsis* (Xie et al., 2009; Sánchez-López et al., 2016). These reports showed the importance of microbial VOCs in affecting different processes and stages of plant growth and health.

17.6 Volatile organic compounds-mediated induced resistance against biotic stress in plants

There is a wide variety of microbial VOCs that are highly toxic to pathogens (Cordovez et al., 2015). These antimicrobial VOCs not only directly inhibit the pathogen growth and its virulence traits (Raza et al., 2016a,b) but indirectly induce systemic resistance in plants against biotic stress (Song and Ryu, 2013). For example, VOCs produced by *Bacillus amyloliquefaciens* T-5 and SQR-9 not only reduced the growth of *Ralstonia solanacearum* but also suppressed its virulence traits (Raza et al., 2016a,b). The VOCs of *Paenibacillus polymyxa* WR-2 decreased the growth and abolished the pigment formation by *Fusarium oxysporum* (Raza et al., 2015). Similarly, a microbial VOC, dimethyl sulfide directly inhibited plant pathogens *Botrytis cinerea* and *Cochliobolus heterostrophus* and indirectly protected tobacco and corn plants by inducing systemic resistance (Huang et al., 2012a,b). A mixed blend of VOCs produced by microbes would play more important role in this regard. For example, 2-undecanal and 2-methyl pentanoate are very lethal to pathogens and on the other hand, some VOCs like acetoin, tridecane induce plant resistance against the invading pathogens (Lee et al., 2012; Ossowicki et al., 2017; Raza et al., 2017). In another report, *Erwinia aerogenes* produced a VOC 2, 3-butanediol, which increased maize growth and induced its resistance against the pathogen *Setosphaeria turcica* (D'Alessandro et al., 2014). Two soil microbes *Bacillus amyloliquefaciens* IN937a and *Bacillus subtilis* GB03 produced a ketone group VOC 2-butanone that induced cucumber systemic resistance against an insect herbivore and a bacterial pathogen (Song and Ryu, 2013). Sharifi and Ryu (2016) reported that induction in systemic resistance by microbial VOCs is the main mechanism to protect plants against pathogen rather than direct pathogen inhibition under natural conditions. Therefore, it is recommended to use a mixed blend of VOCs to have optimum effects which would combine different mechanisms of action.

17.7 Volatile organic compounds-mediated induced resistance against abiotic stress in plants

Another mechanism by which microbial VOCs improve plant growth and health is thought their positive effects on induced resistance against abiotic stress, including drought, temperature, salt, etc. The mechanisms behind this effect includes, Na^+ accumulation, antioxidant capacity, osmoprotectants and modulation of hormone production in plants (Liu and Zhang, 2015; Ngumbi and Kloepper, 2016; Sharifi and Ryu, 2017). For example, VOCs of *Bacillus subtilis* GB03 induced systemic resistance in *Arabidopsis* against salt stress by upregulating high-affinity K^+ transporter 1

(HKT1) in shoots and downregulating in roots (Zhang et al., 2008a). Furthermore, VOCs of *Bacillus subtilis* GB03 increase glycine betaine and choline biosynthesis in *Arabidopsis* (Zhang et al., 2010). Two organic osmolytes, glycine betaine and choline, accumulate in the plant during stress conditions and help plants in enzyme production, membrane integrity and osmotic adjustment (Ashraf and Foolad, 2007). A VOC 2,3-butanediol produced by *Pseudomonas chlororaphis* O6 increased drought tolerance in *Arabidopsis* and NO and salicylic acid were found essential for this activity of VOCs (Chou et al., 2008, 2013). Furthermore, VOC-mediated tolerance to drought stress in plants was achieved through their effect on an essential enzyme (PEAMT) of choline and glycine betaine biosynthesis pathway (Vardharajula et al., 2011).

17.8 Volatile organic compounds-mediated hormone crosstalk in plants

The microbial VOCs mediated effects on plants are mainly driven by their effect on the balance of production of plant hormones. The plants unresponsive or less sensitive to microbial VOCs are not likely to experience the effects of VOCs. For example, VOCs of *Bacillus subtilis* GB03 were less effective on *Arabidopsis*, which was ethylene insensitive 2 (*ein2*) mutant (Ryu et al., 2003). Similarly, VOCs of *Paenibacillus polymyxa* E681 showed an effect in case of mutant plants for salicylic acid, jasmonic acid and gibberellin but not in the case of ethylene insensitive 2 (*ein2*) mutant (Lee et al., 2012). Furthermore, Kwon et al. (2010) showed at the transcriptional level that ethylene biosynthesis and ethylene response genes respond to microbial VOCs. VOCs of *Bacillus subtilis* GB03 increased root proliferation by improving lateral root formation through a pathway require auxin (Zhang et al., 2007). The VOC indole is taken up by plants and act as a precursor for auxin production as reported by many researchers (Bailly et al., 2014; Bhattacharyya et al., 2015). *Pseudomonas vulgaris* increased the growth of *Arabidopsis* seedlings by 50%; however, auxin mutant plants and application of an indole inhibitor (N-1-naphthylphthalamic acid) abolished the effect of VOCs (Bhattacharyya et al., 2015). The microbes *Ampelomyces* sp. and *Cladosporium* sp. produced VOCs, 3-methylphenol and methyl benzoate, that induced systemic resistance in plants against *Pseudomonas syringae* in a jasmonic acid/salicylic acid-dependent fashion. Furthermore, when jasmonic acid, salicylic acid and ethylene mutants were exposed to methyl benzoate, jasmonic acid mutants were not able to resist pathogen. This suggests that jasmonic acid plays an active role. On the other hand, 3-methylphenol induced resistance in a way that require jasmonic acid and salicylic acid. However, ethylene signaling was not employed in both cases (Naznin et al., 2014).

Microbial VOCs-mediated effects on another plant hormone cytokinin, responsible for photosynthesis and flowering, has been reported in several studies (Werner et al., 2001). The VOCs produced by *Alternaria alternata* increased the accumulation of cytokinin in *Arabidopsis* by three folds *in vitro* and this effect resulted in increased photosynthesis and reduced time of floral bud appearance (Sánchez-López et al., 2016). Ryu et al. (2003) reported that *Arabidopsis* mutant for cytokinin receptor-deficient 1 (*cre1*) was unable to respond to the VOCs of *Bacillus subtilis* GB03. In another study, Bhattacharyya et al. (2015) reported that *Proteus vulgaris* VOCs, including indole could not improve plant growth when applied to a *cre1* mutant plant. These results demonstrated above suggest that while fungal and bacterial strains produce different VOCs, plant response to a fungal strain *Alternaria alternata* was very similar to that of a bacterial strain *Bacillus subtilis*. The transcriptomic analysis further showed that around 25% differentially expressed genes were cytokinin-responsive genes (Sánchez-López et al., 2016). As far as abscisic acid is concerned, it produces in plants at the end of photosynthesis as a result of sugar accumulation and negatively regulates the photosynthesis to reduce the accumulation of additional sugar (Rolland et al., 2006; Cho et al., 2010). However, this negative regulation response of abscisic acid was bypassed by the VOCs of *Bacillus subtilis* GB03 and abscisic acid concentration was reduced by 50% in plants exposed to the VOCs compared to untreated control (Zhang et al., 2008b). Though, the exogenous application of abscisic acid abolished the photosynthesis increasing effect of VOCs of *Bacillus subtilis* GB03 (Zhang et al., 2008b). These results demonstrated that different VOCs differentially influence the hormonal cascade of plants and highly conserved signaling network is employed for plant response to microbial VOCs. However, enough information is not available yet to completely unveil the effects of microbial VOCs on plants through their actions on growth-regulating hormones. The further research should focus more on exposing the crosstalk between plant growth regulatory hormones after exposure to the microbial VOCs (Fig. 17.2).

17.9 Volatile organic compounds-mediated recruitment of beneficial insects

Another mechanism by which microbial VOCs help plants to maintain growth and health is the modulation of plant aerial VOCs, which could help plants attract natural enemies of herbivore pests, thereby promoting plant growth. Several reports showed that microbial VOCs not only improved the oil contents of leaves (Zhou et al., 2016) but also

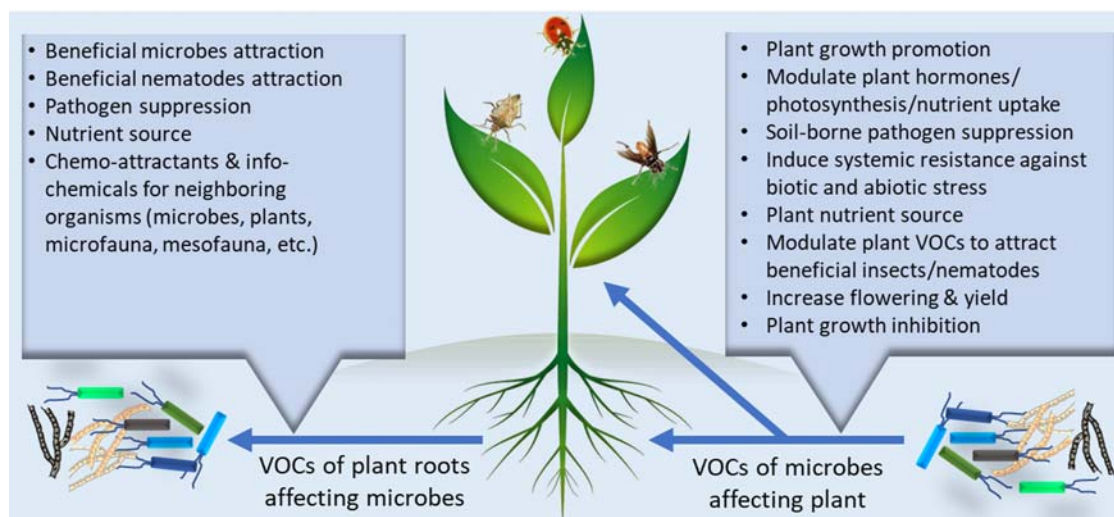


FIGURE 17.2 Effect of volatile organic compounds (VOCs) produced by soil microbes on plants and effect of VOCs of plant roots on soil microbes. While there is plenty of information available describing the effects and mechanisms of VOCs of soil microbes to modulate plant growth and health, the VOCs excreted by plant roots are not very well studied.

the composition of plant VOCs (Sharifi et al., 2018). For example, mycorrhizae change the profile of emitted VOCs in common bean (Schausberger et al., 2012). The plants treated with mycorrhizae *de novo* produced β -caryophyllene and β -ocimene, which attracted the parasitoids of spider mites (Schausberger et al., 2012). In another report, *Arabidopsis* treated with *Pseudomonas simiae* WCS417r showed different VOC profile via jasmonic acid signaling pathway compared to untreated control; however, this change in VOCs profile negatively effected the performance of parasitoids and the sucking insect *Myzus persicae* (Pineda et al., 2013). On the other hand, Pangesti et al. (2015) reported that plants showed more attraction for parasitoids to the chewing insect *Mamestra brassicae* when treated with *Pseudomonas simiae* WCS417r. Under field conditions, cucumber plants treated with two microbial VOCs, pentan-3-ol and butan-2-one showed more recruitment of a natural enemy (ladybird beetles) of aphid pests (Song and Ryu, 2013). In several reports, jasmonic acid was found to be the fundamental regulator of plant VOCs induced by herbivores and microbial VOCs can regulate the signaling pathway and biosynthesis of jasmonic acid (Pineda et al., 2013; Sharifi and Ryu, 2016; Sharifi et al., 2018). The inoculation treatment of aerial parts of plants was also found to modulate the VOC profile of plants; for example, the concentration of plant VOCs (*i.e.*, monoterpenes) was reduced by the leaf inoculation of an endophytic fungus *Neotyphodium uncinatum* (Rostás et al., 2015). While these reports show interesting results of the effect of microbes or their VOCs on the emission of plant VOCs to attract beneficial insects, in-depth studies are not conducted yet. Thus, more work is required to explore the effect of microbial VOCs on plant VOCs and their indirect effect on plant growth and defense.

17.10 Volatile organic compounds-mediated plant growth inhibition

Microbes produce a diverse array of VOCs, among those, while some promote plant growth by the modulation of different aspects of plant physiology as described above, some have no effect on plants. Other than that, microbial VOCs that inhibit plant growth are also reported. Wenke et al. (2012) reported that the VOCs of *Serratia plymuthica* and *Stenotrophomonas maltophilia* showed reduction in *Arabidopsis* plant growth and this effect was mediated by the activation of WRKY transcription factors (*WRKY18*) and defense-related genes in *Arabidopsis*. WRKY transcription factors are involved in many important processes in plants like plant immunity triggered by microbes or pathogen and effector-triggered immunity, response to biotic/abiotic stress, development and physiological processes, etc. (Rushton et al., 2010). However, in another study, WRKY-independent pathways were triggered after exposure to the VOCs (Wenke and Piechulla, 2013). More research is required to unveil the mechanisms behind VOCs-mediated plant growth inhibition and whether production of plant growth inhibiting VOCs is common only in pathogens or other microbes are produced such VOCs.

17.11 Volatile organic compounds released by plant roots

Both monocotyledonous and dicotyledonous plant species have been reported to release plant root VOCs, which mediate plant-plant, plant-microbe, plant-phytophagous pests and plant-parasitic nematode interactions (Ali et al., 2011; Farnier et al., 2012; Palma et al., 2012; Weissteiner et al., 2012). The damage to plants caused by insect herbivores release VOCs, which act as signals for indirect defense and recruit insect predators and entomopathogenic nematodes, similarly, biotic and/or abiotic stresses mediate VOC release by plants as a defense mechanism to recruit beneficial microbes to help plant fight stresses (van Tol et al., 2001; Boff et al., 2002; Rasmann et al., 2005; Ali et al., 2011). Plants produce more than 200,000 metabolites that play important role in the recruitment of beneficial microbes, communication and other ecological interactions below ground. The VOCs blends produced by plant roots mainly belong to VOCs classes like terpenoids, alkanes, sulfur-containing compounds or fatty acid derivatives (Ali et al., 2010; Gfeller et al., 2013; Crespo et al., 2012; Danner et al., 2015). It is estimated that around 1% of plant metabolites are VOCs (Venturi and Keel, 2016), which perform many functions in soil like antimicrobial, food source, chemo-attractants and info-chemicals (Van Dam et al., 2016). Van Dam et al. (2016) reported increase in *Janthinobacterium* and *Pseudomonas* cell numbers after inoculation in a nutrient-poor soil enriched with terpenes suggesting that plant root VOCs act as a nutrient source. Furthermore, VOCs like carvone, limonene, and borneol emitted by plant roots increased bacterial quorum sensing (Ahmad et al., 2015). Legume plant release flavonoids, which attract nitrogen-fixing bacteria (Peters et al., 1986). Similarly, maize plant not only release benzoxazinoids to attract PGPRs such as *Bacillus*, *Pseudomonas*, etc. (Neal et al., 2012) but also a sesquiterpene VOC (E)- β -caryophyllene, which is attacked by root feeding beetle larvae and thus increased the attraction of nematodes towards rhizosphere (Van Tol et al., 2001; Rasmann et al., 2005). In a recent study, it was realized that the migration of soil microbes towards plant roots can be encouraged by the plant roots VOCs. Interestingly, in the presence of pathogen in the rhizosphere, the VOC blend of plant roots was altered and microbes with antifungal activity were attracted (Schulz-Bohm et al., 2018). Furthermore, the addition of nutrients to the bulk soil changed the microbe attraction patterns; for example, attraction for *Burkholderia*, *Dyella* and *Pseudomonas* was reduced in the presence of nutrients in the bulk soil while attraction for *Paenibacillus* was found only with the addition of nutrients in bulk soil (Schulz-Bohm et al., 2018). These recent results suggest that certain microbial species are strongly attracted by plant root VOCs under nutrient-depleted conditions. Hence, plant VOCs as an info-chemical provide information about a nutrient-rich environment nearby. The invasion of pathogen was also found to change the VOC profile of plant roots; for example, the infestation of *Fusarium* spp. triggered a fast emission of sesquiterpenes in cereal crops like maize (Piesik et al., 2011a,b). Similarly, the invasion of *Phytophthora infestans* changed the production of terpenoids by potato plant roots (Becker et al., 2014). These results clearly demonstrate the modulation of plant root VOCs by microbes and their importance in belowground interactions of plants with other organisms including microbes, nematodes etc.; however, in-depth studies are required to get more insight on this topic.

17.12 Research challenges regarding microbial volatile organic compounds

The reported results described above clearly demonstrate that the production of VOCs, which can impact plant growth and health is a widespread feature of soil microbes. The VOCs mediated interactions play important role in the ecology of plants and microbes, but most of the studies are conducted *in vitro* using simple model plant *Arabidopsis* in closed systems. In closed systems, normally whole plant is exposed to VOCs, and thus VOC-plant interactions occur below ground and above ground are undetermined. There are some field studies which show that VOCs might play significant role in plant growth and health under field conditions; however, it requires further open-field experiments to optimize application methods, applicable rate, etc. for large scale application. The model plants should be replaced by economically important plants and greenhouses should be used for initial experiments. In addition, high evaporation rate of VOCs causes inconsistent results which should be addressed. There are also not enough reports available that a single use of VOCs is more effective or their mixed-used would be more effective and what ratio should be used to get optimum output. Furthermore, whether plants should have continuous exposure of VOCs as suggested by Tahir et al. (2017) or a single application would be enough to attain maximum output. Metabolic engineering for microbial strains to improve or expand the production of VOCs can be an important strategy for future application. Most studies are conducted using one to one interactions and information on how microbial communities drives VOCs-mediated ecosystem functioning is still not clear. Furthermore, how plant recognizes or perceive microbial VOCs is still not clear. Can plant block or filter specific VOCs? Or why VOCs like dimethyl disulfide improve growth of one plant but reduce the growth of another? Do plants have specific receptors for VOCs? There are unsolved scientific questions.

17.13 Conclusions

There are considerable reports on the microbial VOCs mediated effects on plant growth and health; however, the information of underlying mechanisms of action is still limited. There are some reports that microbial VOCs interfere with plant signaling pathways, such evidences are still limited to draw a conclusion and we can only depict a hypothetical model of such interactions. Radioactive tracer studies using synthetic VOCs should be done to uncover that how VOCs act as signals, enter and transported throughout host plant and activate signaling cascade. The important elucidated VOCs should be tested under conditions near to field situations. A deeper understanding, describing concentration of VOCs, environmental conditions and mechanisms of action would highlight the commercial potential of microbial VOCs as a cost-effective, environmentally friendly alternative to chemicals as a next-generation pesticides and fertilizers or both. The field of VOCs-mediated interactions in different organisms is still infancy, but it holds great promise for future discoveries.

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Chapter 18

Molecular mechanisms in plant growth promoting bacteria (PGPR) to resist environmental stress in plants

Udaya Kumar Vandana, Bijoya Singha, A.B.M. Gulzar and P.B. Mazumder

Department of Biotechnology, Assam University, Silchar, India

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18.1 Introduction

Abiotic stresses involve non-living factors that establish negative impact on living organisms in a particular environment. Studies have also shown that abiotic stresses are more harmful when they occur in combination (Mittler, 2006). Drought, heat, salinity, cold, or heavy metal stress can cause deplorable effects on plant growth and yield under field conditions. Such environmental stress can have alterations in the physiological traits in plants. However, currently impacts of such stresses on plants are usually being studied under controlled growth conditions in vitro. Noted that the field environment differs largely from the controlled conditions in laboratory, often involving plants, simultaneously, exposed to more than one abiotic and /or biotic stress conditions, combination of such as drought and cold, drought and heat, heat and salinity, or any of the major abiotic stresses with metal stress. Circumstances under combined stresses are largely controlled by a high degree of different signaling pathways which can interact and inhibit each other.

Plant root system is circumvented with a thin layer of soil, termed as rhizosphere which aids in providing an active area for root activity and metabolism. Bacteria are the most abundantly found microbes surrounding the rhizosphere. Plant growth promoting rhizobacteria (PGPR) are known to have a positive interaction with plant roots which influence the plant growth from direct mechanisms to indirect effects. Unique properties of microorganisms can be explored which include tolerance to extremities such as biotic and abiotic stress, their genetic diversity, and their synergy with crop plants. This assists in establish methods for their disposition in agriculture production providing skillful methods to understand the adaptation, stress tolerance, and response mechanisms, which can be later engineered into crop plants to study their confrontation with stresses. The main objective of this chapter is to review environmental stress; especially drought and salinity stress and the influence of PGPR, as possible antagonists, on crop plants under such stress conditions. Emphasis was given on how the harmful chemical fertilizers and how they can be replaced with the use of PGPR in stress affected areas (Fig. 18.1).

18.1.1 Drought stress

Drought is one of the most destructive abiotic stresses, a general term that describes the atmospheric or weather phenomena (Shao et al., 2008; Jaleel et al., 2009; Dai, 2013). Mainly, drought stress occurs when there is a decline in level of available water for plants in soil due to sparse soil moisture at a specific time (Keyvan, 2010; Dai, 2013). Furthermore, when the transpiration rate in the leaves surpasses the water level in plant can lead to water stress (deficiency). Water aptitude of plant roots is higher than that of the soil, ultimately, leading to such imbalance in water uptake and water losses from plants. Drought stress has elevated in intensity over the past decades affecting world's food security in large scale. Drought stress restricts crop yields, which may range from moderate and comprehensive to excessively intense and extended duration, which is expected to impact serious plant growth problems for more than 50% of the arable lands. It may occur in almost areas, especially arid and semiarid regions.

18.1.2 Effect of drought stress on crop plants

18.1.2.1 Nutrient diffusion

Drought induces plant-water turgor potential, abundantly interfering with normal functions. Drought stress has been studied extensively to reduce the growth in several crops viz maize (Kamara et al., 2003), barley (Samarah, 2005), wheat (Rampino et al., 2006), and rice, leading to decrease in nutrient diffusion and bulk flow of water-soluble nutrients such as calcium, magnesium, silicon, nitrate, and sulfate (Barber, 1995; Selvakumar et al., 2012). Generally, nutrients are carried to the roots by water, drought stress is known to impact on the availability and transport of nutrients.

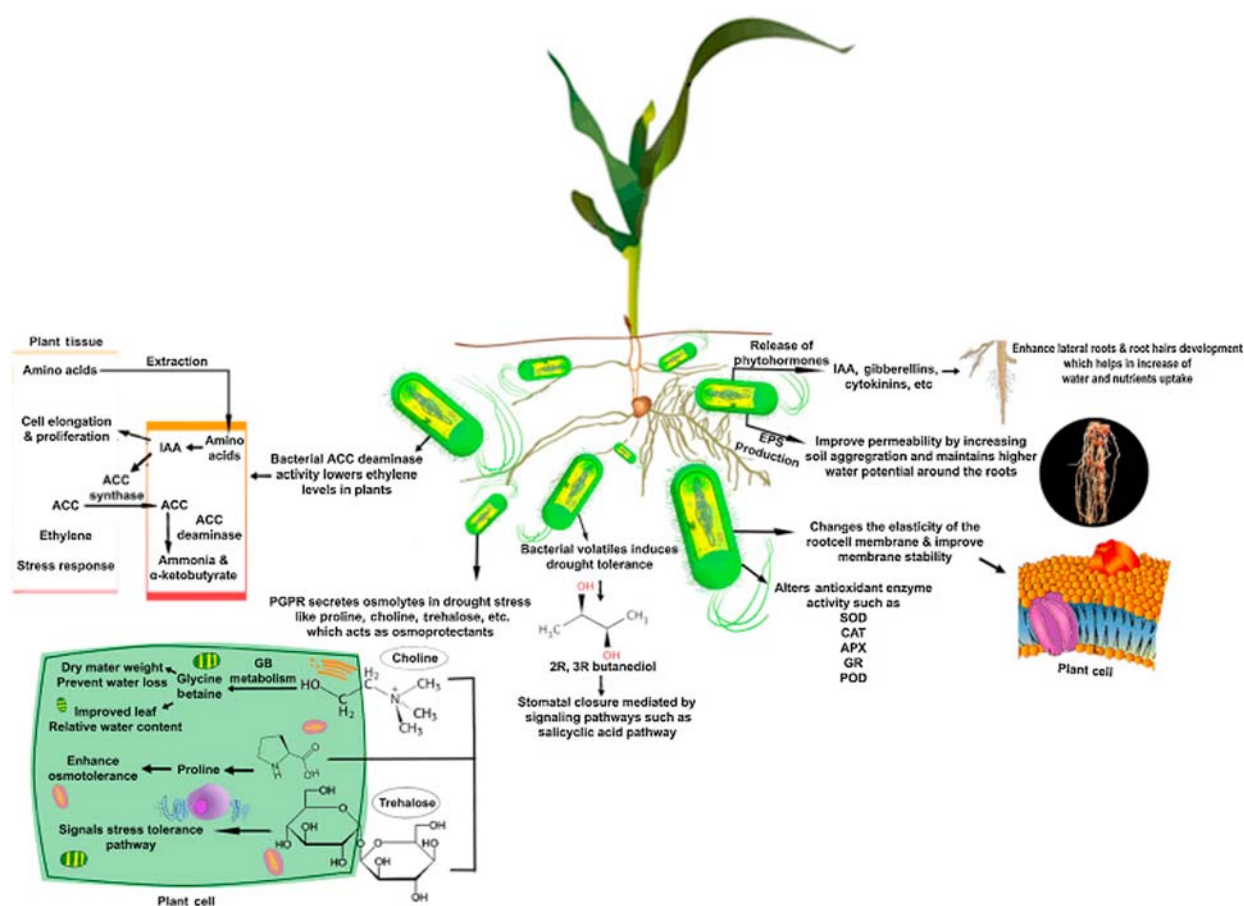


FIGURE 18.1 Mechanism of plant drought tolerance induced by PGPR (Vurukonda et al., 2016).

18.1.2.2 Morphological and anatomical effects

Drought can influence morphological and anatomical characteristics of plants in many facets. Water deficiency alters the anatomy of a leaf and its ultrastructure (Rao et al., 2006; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Consequences such as diminishing of leaves' size, reduced aperture and declining in number of stomatal cells, thickening of cell wall, leaf surface cutinization, and development of conductive system i.e. augmentation in number of large vessels, stomatal submergence in xerophytic plants, and formation of tube leaves were some of the alterations been extensively studied in plants which were exposed to drought (Chernyad'ev, 2005; Shao et al., 2008; Jaleel et al., 2009; Keyvan, 2010; Mafakheri et al., 2010; Nezhadahmadi et al., 2013; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). The vital aspects to carry out optimal photosynthesis in plants involve optimal leaf area development and stomatal opening. Therefore, water deficiency in plants, due to low leaf area, a higher resistance for exchange in stomata, and an elevation in leaf senescence, net photosynthesis is reduced (Shao et al., 2008; Mishra and Singh, 2011; Zare et al., 2011). A typical symptom seen to be observed under drought stress is decrease in chlorophyll content that could totally alter plant morphology (Mishra and Singh, 2011; Rahdari and Hoseini, 2012; Arbona et al., 2013; Bhargava and Sawant, 2013; Fahraji et al., 2014). Plants extend their roots in order to raise water uptake under dehydrated conditions, and to develop ramified root system (Farooq et al., 2009; Jaleel et al., 2009; Rahdari and Hoseini, 2012; Akhtar and Nazir, 2013; Bhargava and Sawant, 2013).

18.1.2.3 Photosynthesis

One of the main effects of droughts in higher plants involves reduction and/or inhibition photosynthesis (Keyvan, 2010; Nezhadahmadi et al., 2013; Bhargava and Sawant, 2013). Under water inadequacy, stomatal and/or non stomatal impact can be effective in inhibiting photosynthesis (Zlatev and Lidon, 2012). Thus, due to prolonged stomatal closure, there are limitations in CO₂, especially under conditions such as light saturation conditions which lead to augmentation of diminished photosynthetic electron transport components. Such accumulation of components may lower molecular O₂, giving rise to production of reactive oxygen species (ROS) including superoxide and hydroxyl radicals, thereby causing oxidative damage in chloroplasts (Shao et al., 2008; Zlatev and Lidon, 2012; Bhargava and Sawant, 2013; Arbona et al., 2013; Osakabe et al., 2014; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Furthermore, reduced uptake of carbon dioxide decreases the rate of photosynthesis due to reduced activity of enzymes in CO₂ reduction such as in C₃ cycle (Calvin cycle), dark reactions. The fundamental enzyme in the C₃ cycle for carbon metabolism is ribulose-bisphosphate carboxylase (RuBisCO). Rate of biosynthesis controls the level of RuBisCO in leaves, where RuBisCO amount and activity declines rapidly under water deficit conditions. Such decline of RuBisCO activity occurs due to some of the main attributes such as acidification of chloroplast stroma, structural alterations of chloroplasts and RuBisCO, a reduction activity of coupling factor (ATPase, ATP synthase), damaged plastids releasing RuBisCO (Chernyad'ev, 2005; Farooq et al., 2009; Zlatev and Lidon, 2012; Akhtar and Nazir, 2013; Bhargava and Sawant, 2013; Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

18.1.2.4 Hormonal balance

Hormones are known to play key roles in regulating plant processes, of which some are involved in plant interactions with environmental stresses such like droughts (Bernacchia, Furini, 2004; Fahraji et al., 2014). Among all the plant hormones, the most effective hormone in response to drought stress is ABA (Bernacchia and Furini, 2004; Bhargava and Sawant, 2013; Akhtar, and Nazir, 2013). After exposure of plants to drought, ABA synthesis in roots takes place which is translocated to shoots, especially leaves. The translocation of ABA to stomata urges stomatal closure, decreasing stomatal conductance capacity. One of the earliest plant responses is the higher stomatal resistance which leads to reduced water losses from the leaf surface. Auxins be contrary to ABA; they act as negative regulators of water loss resistance as indole-3-acetic acid (IAA) down regulation aids in aggregation of late embryogenesis abundant mRNA. Under water deficit stress condition, cytokinin (zeatin) and gibberellin (GA₃) levels reduce rapidly. In some studies, cytokinins show delayed senescence, thereby leading to better adaptation of plants by delaying drought-induced senescence (Chernyad'ev, 2005; Bhargava and Sawant, 2013). In case of brassinosteroids, they are known to raise water uptake and cell membranes, and also under drought stress they are known to reduce ion leakage from membrane (Rahdari and Hoseini, 2012; Bhargava and Sawant, 2013).

18.1.3 Influence of PGPR under drought stress

The role of PGPR in plant growth development is significant in establishing nutrient management and biocontrol activity, by colonizing the rhizosphere of plants via numerous direct and/or indirect mechanisms (Grover et al., 2011). The role of rhizobacteria is gaining importance in regulating against biotic and abiotic stresses. Therefore, there are various mechanisms that operate in microbes possessing stress ameliorating potential and their subsequent use in sustainable agriculture.

18.1.4 Role of PGPR in modification of phytohormonal activity

Plants produce phytohormones such as indole-3-acetic acid (IAA), gibberellins (GAs), ethylene, abscisic acid (ABA), and cytokinins, crucial for plant growth and development (Barea and Brown, 1974; Frankenberger and Arshad, 1995; Egamberdieva and Kucharova, 2009), which are shown to have abiotic stress tolerance. In addition, phytohormones can be synthesized by PGPRs that can stimulate plant cell growth. Physiologically, IAA is the most active auxin in plant growth and development. Dimkpa (2009) studied various plant species when inoculated with IAA-producing bacteria have shown to increase root growth and/or augment lateral roots and root hairs formation. This leads to increase in water and nutrient uptake (Mantelin and Touraine, 2004), assists plants to cope with water deficit (Egamberdieva and Kucharova, 2009). Inoculation of *Solanum lycopersicum* (tomatoes) with *A. brasilense* producing nitric oxide (a small diffusible gas) acts as a signaling molecule in IAA inducing pathway and enhances adventitious root development (Creus et al., 2005; Molina-Favero et al., 2008). Likewise, inoculation of *Phaseolus vulgaris* L. (common bean) with *A. brasilense* has been observed to increase root projection area, specific root length, and specific root area (German et al., 2000). Cohen et al., 2009 studied in maize plants that *Azospirillum lipoferum* produce ABA and gibberellins which alleviate drought stress. The dehydration in cells results in induction of biosynthesis of ABA which is a stress hormone released during water deficit condition (Kaushal and Wani, 2016). ABA is also being observed to be involved in water-loss regulation by controlling stomatal closure and stress signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki, 1994).

18.1.5 Role of ACC deaminase as a drought stress ameliorating agent

Hardoim et al., 2008 hypothesized that levels of ethylene are responsible for regulating plant activities and the biosynthesis of ethylene levels is regulated by biotic and abiotic stresses. The mechanisms include drought responsive genes expression and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. In ethylene biosynthesis pathway, 1-aminocyclopropane-1-carboxylate synthase (ACS) converts S-adenosylmethionine (S-AdoMet) to 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene. Under stress conditions, such as drought, plant homeostasis is regulated by ethylene which ultimately results in reduced root and shoot growth. Nonetheless, deterioration of the ethylene precursor ACC by bacterial ACC deaminase results in releasing plant stress, ultimately rescuing normal plant growth. The ethylene production under high salt stress. Mayak et al., 2004 observed that ACC deaminase, which produces PGPR *Achromobacter piechaudii* ARV8, notably increased the fresh and dry weights of both pepper and tomato seedlings, and the ethylene production was reduced under drought stress.

18.1.6 Molecular studies in amelioration of drought stress by PGPR

Understanding and comparing an organism's "holistic" responses with respect to their environment are accomplished by studying their gene expression. The most appropriate way to carry out such studies include assaying the transcriptome that consists of the entire set of transcripts (expressed within a cell at a specific developmental stage). Various technologies include hybridization-based microarrays to RNA sequencing (Trewavas, 2006; Wang et al., 2009). It was observed that at the transcriptional level, PGPR enhanced drought stress tolerance of *Arabidopsis thaliana* when inoculated with PGPR *Paenibacillus polymyxa* B2. Timmusk and Wagner (1999) showed in their studies that inoculated plants display mRNA transcription of a drought-response gene, EARLY RESPONSE TO DEHYDRATION15 (ERD15) when compared to uninoculated controls.

Methods such as 2-D polyacrylamide gel electrophoresis (2D-PAGE) and differential display polymerase chain reaction (DD-PCR) aid in identification of six differentially expressed stress proteins in pepper plants when inoculated with *B. licheniformis* K11 under drought stress. Lim and Kim (2013) described that among the stress proteins, specific genes of Cadhn, VA, sHSP and CaPR-10 showed more than a 1.5-fold increase in treated plants when compared to untreated

controls. Real-Time PCR (RT-PCR) was applied to identify the upregulation of stress related genes; APX1, SAMS1, and HSP17.8 in the leaves of wheat alongwith elevated enzymatic activity involved in the plant ascorbate glutathione redox cycle conferring drought tolerance in wheat having primed with *Bacillus amyloliquefaciens* 5113 and *A. brasiliense* NO40, ameliorating the deleterious effect of drought stress (Kasim et al., 2013). Approaching microarray analysis was also one of the molecular aspects in which a set of drought signaling response genes were down regulated in the *P. chlororaphis* O6-colonized *A. thaliana* when compared to those without bacterial treatment under drought stress (Table 18.1).

18.2 Salinity stress

The high level of salt into the soil prevents the plant attaining full genetic potential and reduces plant growth and development which ultimately decreases the crop yield and the quality of the grain (Atkinson and Urwin, 2012). Depending on the response to the salt concentrations plants are classified into two groups (1) Halophytes: plant that can survive under high salt concentration i.e. salt tolerant plant. (2) Glycophytes: salt sensitive plants. Majority of the crop plants are fall under the second category (Gupta and Huang, 2014). And thus, salinity stress is one of the major reasons to decrease the crop yield. High salt concentration into the soil initiates osmotic stress, ionic stress, and oxidative stress into the plants which ceases the productivity of the plant (Rahneshan et al., 2018). Due to the hypertonic condition of the soil, plant cell accumulate toxic ion (Na^+ and Cl^-) inside the cell which results in water imbalance and ion homeostasis (Ilanguvaran and Smith, 2017). Salinity hampers various physiological and metabolic processes which reduces plant growth and development. Changes in physiological and metabolic processes reduces leaf area, increases leaf thickness, necrosis in roots, and reduces internode length (Rahneshan et al., 2018).

18.2.1 Plant response under salinity stress

Excess amount of salt concentration into the soil disturbs the soil fertility (Ilanguvaran and Smith, 2017). Due to the hypertonic condition of the soil, osmotic stress is the immediate impact to the plants. Plant cell accumulates various toxic ion (Na^+ and Cl^-) inside the cell from the soil (Munns, 2005). Toxic ions disturbs the water balance, and ion homeostasis in the plants, which affects in hormonal regulation, transpiration, photosynthesis, nutrient uptake, and other metabolic processes (Ilanguvaran and Smith, 2017). Plant roots first sense the salinity stress and generate a hormonal signal and send it to the shoot apical meristem which decreases the plant shoot growth and reduces photosynthetic leaf area (Munns, 2002). Accumulation of excess level of salt from the soil leads to the premature leaf senescence and reduce the photosynthetic leaf area of the plant which ultimately affect the plant growth (Munns 2005).

TABLE 18.1 PGPR inducing drought responsive genes in plants (Vurukonda et al., 2016).

PGPR	Plant species	Drought responsive genes	Reference
<i>Paenibacillus polymyxa</i> B2	<i>Arabidopsis thaliana</i>	Induction of EARLY RESPONSE TO DEHYDRATION 15 (ERD15)	Timmusk and Wagner (1999)
<i>B. licheniformis</i> K11	Pepper	Inoculation increased stress protein genes Cadhn, VA, sHSP and CaPR-10	Lim and Kim (2013)
<i>Bacillus amyloliquefaciens</i> 5113 and <i>Azospirillum brasilense</i> NO40	Wheat	Upregulation of stress related genes APX1, SAMS1, and HSP17.8	Kasim et al. (2013)
<i>Pseudomonas chlororaphis</i> O6	<i>Arabidopsis thaliana</i>	Transcripts of the jasmonic acid-marker genes, VSP1 and pdf-1.2, salicylic acid regulated gene, PR-1, and ethylene-response gene, HEL, were up-regulated in colonized plants	Cho et al. (2013)
<i>Gluconacetobacter diazotrophicus</i> PAL5	Sugarcane	Inoculation activated the ABA-dependent signaling genes conferring drought resistance	

18.2.1.1 Role of salinity in seed germination

Seed germination is the first phase of the growth cycle in plants (Parihar et al., 2015). Salinity adversely affects seed germination, excess amount of soluble salt content into the soil reduces the water potential into the soil. As water moves from higher water potential to lower water potential, seeds are unable to take water from saline soil and causes hormonal imbalance (Khan and Rizvi, 1994), reduces protein metabolism (Dantas et al., 2007), nucleic acid metabolism (Gomes-Filho et al., 2008) and ultimately reduces the utilization of seed reserves (Othman et al., 2006). There are some evident that salinity drastically affects the seed germination in various plants like *Oryza saliva* (Xu et al., 2011), *Triticum aestivum* (Akbarimoghaddam et al., 2011), *Zea mays* (Khodarahmpour et al., 2012), *Brassicasp.* (Akram and Jamil, 2007). Bybordi (2010) reported that with the increasing salt concentration the rate of seed germination decreases in *Brassica napus* (Bybordi, 2010).

18.2.1.2 Role of salinity in plant growth

Under salt stress plant growth is mainly inhibited by two mechanisms. First, osmotic or water defect effect of salinity. In this case excess amount of salt concentration into the soil reduces the water potential into the soil and hence plants are unable to uptake water from soil and reduces plant growth. Second, ion toxicity effect of salinity. In this case plant cell accumulates excess ions inside the cell from the soil, these ions may enter into the transpiring cell and may damage the cell and decreases plant growth (Parihar et al., 2015).

18.2.1.3 Salinity and ion toxicity in plants

High concentration of soluble salt into the soil competes with the nutrients and minerals which are essential for various metabolic processes into the plants (Parihar et al., 2015). Appropriate ion concentration into the soil plays crucial role for plant growth and development (Wang et al., 2003). Increased salt concentration into the soil decreases the uptake of essential nutrient like potassium (K^+), phosphorus (P^+), calcium (Ca^{2+}), and nitrogen (N) (Zhu, 2001).

According to Gadallah (1999), salinity into the soil increases Na^+ content into the *Vicia faba*, while Na^+/K^+ ratio decreased, suggesting relationship between Na^+ and K^+ . i.e. increased Na^+ ion concentration decreases K^+ ion uptake from the soil into the plants (Gadallah, 1999).

According to the Khan et al. (2000) salinity stress showed positive relationship between Na^+ and Cl^- into *Atriplex griffithii* plants. Highest accumulation was found in leaves followed by stem and roots. Increased concentration of Na^+ and Cl^- decreases the concentration of Ca^{2+} into the plants showed negative relationship between Na^+ , Cl^- and Ca^{2+} . Mg^{2+} was not affected into the stem and roots of the plants but the concentration was reduced into the leaves with the increased level of Na^+ and Cl^- (Khan et al., 2000).

It has been reported that the chlorophyll content into the plant decreases as the concentration of the Ca^{2+} and Mg^{2+} decreases due to the salt stress (Parida et al., 2004).

Tavakkoli et al. (2011) separately studied the effect of Na^+ , Cl^- and NaCl stress in barley plants. He reported that the reduction in plants are greater under NaCl stress as compared to Na^+ and Cl^- stress (Tavakkoli et al., 2011). He also reported that the high concentration of Na^+ reduces the uptake of K^+ and Ca^{2+} from the soil and reduces photosynthetic rate by reducing metal conductance, while excess amount of Cl^- leads to the chlorophyll degradation and alters in the photosynthetic capacity into the plants (Tavakkoli et al., 2011).

18.2.1.4 Photosynthesis under salinity stress

The growth and development of a plant depends on photosynthesis. Even a minute change in photosynthetic rate affects in plant growth (Parihar et al., 2015). Presence of soluble salt into the salt reduces the water potential, which is the main reason for the reduction of photosynthetic rate under salinity stress. Chlorophyll is the main component of photosynthesis and it is used as an indicator to determine plant health. During salinity stress Na^+ and Cl^- ions are accumulated into the chloroplast and degrade chlorophyll and hence the rate of photosynthesis decreases drastically (Zhang et al., 2005). There are many reports that during the salt stress the chlorophyll content of the plant decreases and hence chlorophyll content of plant is used as an indicator to determine cellular metabolic state of a plant (Chutipaijit et al., 2011).

Amirjani (2011) reported that the chlorophyll a and chlorophyll b level in *Oryza sativa* reduced under NaCl treatment. He also found that under NaCl treatment the level of chlorophyll b content was affected more as compared to chlorophyll a (Amirjani, 2011). In another study Chutipaijit et al. (2011) reported that the *Oryza sativa* plant treated with 100 mM NaCl showed reduction in chlorophyll a, chlorophyll b, and carotenoid level (Chutipaijit et al., 2011).

Khan et al., 2013 in her experiment on cucumber found a negative relationship between NaCl and total chlorophyll content in cucumber leaf. Khan et al. (2013) reported that with the increasing level of NaCl the total chlorophyll content in cucumber leaves decreased significantly (Khan et al., 2013). According to the Mane et al. (2010), membrane deterioration is the main reason to decrease the pigment level, and decrease in chlorophyll level under stress condition is now the common phenomena (Mane et al., 2010). Allakhverdiev et al. (2000) in his study reported that the NaCl stress affects oxygen evolving complex of photosystem II (PS II) and reduces rate of photosynthesis (Allakhverdiev et al., 2000). Stepien and Klobus (2005) reported that under salinity stress the efficiency of PS II, electron transport chain (ETC) and CO₂ assimilation decreases and alters in plant growth and development (Stepien and Klobus 2005). Lopez-Climent et al. (2008) reported that salinity stress induces stomatal conductance as a result the photosynthetic rate, CO₂ assimilation in the plant reduced and alter in plant growth (Lopez-Climent et al., 2008).

18.2.1.5 Salinity and oxidative stress

Excessive salt stress leads to the stomatal closer, which decreases the CO₂ accumulation into the leaves. As the CO₂ level decreased in the leaves leads to the generation of excessive ROS such as hydrogen peroxide (H₂O₂), Super oxide (O₂*⁻), hydroxyl group (OH*⁻), and singlet oxygen (Parihar et al., 2015). On the other hand, salt stress leads to the water deflect into the plant which ultimately leads to the ROS generation into the plants (Elstner, 1987; Cheeseman, 1988). As ROS leads to the lipid peroxidation, oxidation of protein, inactivation of enzyme, and DNA damage and alter into the metabolic processes of plants and prevents plant growth and development (Parihar et al., 2015).

18.2.2 Influence of PGPR under salinity stress

There are many reports that inoculation of PGPR during the abiotic stress condition showed positive result via direct and indirect mechanism that induce systemic tolerance (Yang et al., 2009). Many PGPR have been found to play important role in improving plant water relation, ion homeostasis, photosynthetic efficiency under salt stress. PGPR also found in relation to stomatal conductance, ion transport, nutrient uptake, carbohydrate metabolism, hormonal regulation, etc. which play important role in the determination of induced systemic tolerance (Ilangumaran and Smith, 2017).

18.2.2.1 Role of PGPR in osmotic balance

During stress condition PGPR are found to regulate water potential and stomatal opening, water potential affects hydraulic conductivity and the transpiration rate in plants (Zhu, 2001). According to Marulanda et al. (2010) Maize plant inoculated with *Bacillus megaterium* showed increased level of hydraulic conductivity of plant root and increased the expression of the plasma membrane aquaporin protein as compared to the uninoculated plants (Marulanda et al., 2010). PGPR helps the plant in the accumulation osmoprotectant and phytohormone signaling and facilitate the plants to overcome from the salinity stress (Chen et al., 2007).

There are many evidences that plant inoculated with salt tolerant PGPR helped the plants to tolerate under salinity stress. According to the Nautiyal et al. (2013), rice plant inoculated with *Bacillus amyloliquefaciens* SN13 under salt stress in both hydroponic and soil condition in greenhouse showed better result against salt stress. Inoculation of SN13 altered the expression of 14 genes, out of which 4 genes (*SOS1*, ethylene responsive element binding proteins *EREBP*, somatic embryogenesis receptor-like kinase *SERK1* and NADP-malic enzyme *NADP-Me2*) were found upregulated while 2 genes [glucose insensitive growth (*GIG*) and (*SNF1*) serine-threonine protein kinase *SAPK4*] were downregulated under hydroponic condition, while only one gene *MAPK5* (Mitogen activated protein kinase 5) was upregulated in soil condition in greenhouse (Nautiyal et al., 2013). PGPR were also found in carbohydrate metabolism and transport, which was the key feature of source sink relationship and enhanced photosynthesis, growth rate of the plant (Dodd and Pérez-Alfocea, 2012).

During salinity stress soluble salt concentration increases into the soil and hence water potential decreases and alters in seed germination. Wheat seed treated with *Bacillus aquimaris* showed increased level of soluble sugar and reducing sugar and facilitated the seeds in imbibition under salinity stress. Inoculated seeds showed increased shoot biomass NPK accumulation and decreased Na accumulation into the leaves (Upadhyay and Singh, 2015). *Capsicum annum* treated with *Azospirillum brasilense* and *Pantoea dispersa* showed increased dry weight (DW) in roots under salinity stress. The Cl⁻ concentration decreased and NO₃⁻ concentration, along with K⁺/Na⁺ ratio were increased as compared to the control and related to the enhanced stomatal conductance and photosynthesis, but the chlorophyll concentration and the photosystem II were unaffected by the inoculant (del Amor and Cuadra-Crespo, 2012).

Under stressed condition PGPR plays important role in the biosynthesis and the accumulation of osmoprotectant like proline, trehalose and glycine betaines into the cytosol, and maintains osmotic balance which prevents oxidative cellular damage under saline condition (Ilangumaran and Smith, 2017). According to the Figueiredo et al. (2008), bean (*Phaseolus vulgaris*) plant inoculated with *Rhizobium tropici* and *Paenibacillus polymyxa* were found in the overexpression of trehalose 6-phosphate gene, which resulted in increased nodulation, N content in plant and showed positive result in plant growth and development as compared to the control under salinity stress.

18.2.2.2 PGPR in ion homeostasis

Microbes play important role in ion homeostasis during the stress condition and helps the plant to tolerate in harsh condition. During salinity stress soluble salt concentration increases into the soil and decreases the water potential. As water moves from higher water potential to lower water potential, plants unable to uptake water from the soil which results in water deflect into the plant (Dodd et al., 2010).

High salt concentration also competes with the nutrient into the soil and reduces the nutrient uptake. Plant also accumulates toxic ion Na^+ and Cl^- into the root cell which are then transported into the leaves and alter in stomatal conductance, CO_2 assimilation into the leaves and decreases photosynthetic rate (Ilangumaran and Smith, 2017).

Accumulation of toxic ion from the soil were reduced by microbes either by altering host physiology i.e. by regulating ion transporter activity, or by forming physical barrier around the root by secreting exopolysaccharide. Microbes also play direct role in the reduction of toxic ion accumulation but improves the nutritional status by increasing uptake of both micro and macro nutrient under salinity stress (Dodd and Pérez-Alfocea, 2012). Nutrient uptake is also enhanced by microbes, as microbes changes the rhizosphere pH by organic acid excretion. Microbes also play important role in organic molecule chelation via siderophore production (Dodd and Pérez-Alfocea, 2012; Ilangumaran and Smith, 2017).

Zuccarini and Okurowska (2008) reported that beneficial microbe and plant interaction increases K^+/Na^+ ratio under salinity stress (Zuccarini and Okurowska, 2008). Exopolysaccharides secreted by PGPR interacts with the cations like Na^+ into the roots and reduced the Na^+ accumulation into the leaves. The exopolysaccharides act as a physical barrier in root surface and reduced the apoplastic flow of sodium ion (Ashraf et al., 2004). 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) secreted by PGPR increased N, P, and K uptake, which results in higher accumulation of K^+/Na^+ ratio into the leaves of maize under salinized condition (Nadeem et al., 2009). PGPR were also found to increase P, K^+ , and Ca^{2+} uptake into the tomato plant (Mayak et al., 2004).

18.2.2.3 PGPR mediated phytohormonal regulation under salinity stress

Plant hormonal status is modulated by PGPR via secreting various exogenous hormones, metabolites and various enzymes and that may help the plant to survive under salt stress. Besides the secretion of hormones, metabolites etc. plant microbe interaction also play important role in various signaling processes (Dodd et al., 2010).

Under salinity stress the ABA concentration increased in root, xylem sap, and shoot, as a result the transpiration rate is decreased (Dodd and Pérez-Alfocea, 2012). During salinity stress the cucumber plant inoculated with *Burkholderia cepacia* SE4, *Promicromonospora* sp. SE188 and *Acinetobacter calcoaceticus* SE370 showed increased biomass. Increased level of water potential and decreased electrolyte leakage were also reported in inoculated plants under salinity stress. Inoculated plants showed down regulation of ABA as compared to the uninoculated plants while the level of salicylic acid (SA), Gibberellin concentration were increased (Kang et al., 2014). Cotton seeds inoculated with *Pseudomonas putida* Rs198 strain showed increased germination percentage and increased level of plant biomass as the concentration of ABA was reduced and endogenous IAA level was increased (Yao et al., 2010).

The ABA and ACC content is also decreased when the wheat plant is inoculated with the PGPR strain *Arthrobacter protophormiae* SA3 and *B. subtilis* LDR2 (Barnawal et al., 2017). PGPR plays important role in auxin biosynthesis, one of the important mechanisms is utilization of tryptophan from the root exudates and conversion into IAA and is absorbed by plant roots (Dodd et al., 2010). Along with the endogenous IAA plant utilize the exogenous IAA produced by PGPR and trigger the auxin signaling pathways which stimulates plant growth and proliferation (Spaepen and Vanderleyden, 2010).

Xie et al. (1996) designed an experiment to understand the specific role of IAA production in PGPR mediated growth promotion. A mutant of *Pseudomonas putida* Gr12-2/aux1 strain having four-fold of IAA producing ability than the wild type, lost the ability to induce root elongation (Xie et al., 1996). But the growth rate, siderophore production, and ACCD activity remain unaltered. During stress condition plant secretes excess amount of ethylene for the adaptation in stress condition with the expanse of growth and development (Ilangumaran and Smith, 2017). Excess amount of

ethylene reduces root elongation, and premature leaf senescence and alter the plant growth (Dodd and Pérez-Alfocea, 2012).

There are many reports that PGPR having ACCD activity play important role in growth promotion under salt stress. Tomato plant treated with *Pseudomonas putida* UW4 strain having ACCD activity showed increased shoot growth under salinity stress (Yan et al., 2014).

Pea plant treated with *Variovorax paradoxus* 5C-2 showed increased K^+ flow from root to shoot, and deposition of Na^+ into the roots. Inoculation with PGPR also increased the photosynthetic rate, electron transport, ultimately increased the plant biomass (Wang et al., 2016).

18.2.2.4 Molecular mechanism in some plants under drought stress

Transgenic plants are approached by plant scientists not only to enhance quality and yield but also to increase tolerance to biotic and abiotic stresses in numerous crops. Nowadays, plant biotechnologists are focusing much on molecular level through genetic engineering on genes encoding ion transport proteins, compatible solutes, antioxidants, heat shock, and late embryogenesis abundant proteins and transcription factors for gene regulation. Tarczynski 1992 mentioned about microbial genes that have been frequently used to engineer traits involved in stress tolerance, transgenic tobacco plants carrying bacterial gene coding for mannitol-1-phosphatodehydrogenase (mt1D) accumulated mannitol in their cytoplasm and were with improved biomass growth under salt stress. Transgenic plants when transformed with *imt1* gene encoding for myo-inositol-omethyltransferase enzyme involved in the ononitol biosynthesis were more drought and salt tolerant as compared to the wild-type plants. The structural gene *TPS1* encodes for trehalose biosynthesis in yeast catalyzed by a key enzyme trehalose-6-phosphate synthase. Gene of *S. cerevisiae* was fundamentally expressed in transgenic potato plants, where the *TPS1* transgenic potato plants have been observed to give significantly enhanced drought resistance.

Genetic engineering has also been used successfully in rice plants to manipulate its abiotic stress tolerance by over expression of *E. coli* trehalose biosynthetic genes (*otsA* and *otsB*).

Identification of transcription factors and regulatory genes in stress-inducible expression of osmoprotectant biosynthetic pathways are also now greatly concerned, for which tools including vectors for multiple gene transfer, stress-inducible promoters and effective selectable markers will be needed to be developed and evaluated.

PGPR with miscellaneous genera are reported to have a large numbers of specific proteins which showed to increase in the expression level upon disadvantageous conditions such as salinity stress.

Proteomics which is the large-scale study of proteomes, is one of the best strategies used to expose the vital whole protein expression in cellular interaction. 2D PAGE when combined with high throughput mass spectrometry and bioinformatics (computational biology), allows identification of unknown proteins by carrying out protein separation and identification which aid in ascertaining functions of genes and their response to analogous physiological actions.

18.3 Future perspectives

Substantial reduction in the use of chemical fertilizers and pesticides is a major concern by developing efficient microbial formulation to boost plant performance under drought stress. Research on using isolated indigenous PGPR from the stress-affected soils that could be used as bio inoculants for crops grown in stressed ecosystems should be focused (Kaushal and Wani, 2016). Many molecular techniques are becoming available recently, and is being applied to define the molecular bases of the plant-microbiome interactions and is still in progress to acknowledge the understanding the mechanism of PGPR imparting drought tolerance in plants, for what we need a better understanding of beneficial PGPR interaction with plants and handling microbes. Nonetheless, current progress in this area disclose the fact future research has great potential to provide new intuitions for sustainable food production.

Future possibility in terms of salinity stress involve inoculation of salt tolerating PGPR into plant cell suspension, regenerating embryos and salt tolerating plants, in tissue culture laboratory. A promising future application could be the improvement of drought and/or salt tolerance by implication of transgenic plants by identifying enzymes and genes involved in the synthesis of osmoprotectants which are found in stress-tolerant microorganisms which are expected to fulfill such opportunities engineering self-tolerance in agricultural crops.

18.4 Conclusion

Drought stress is one of the most devastating constraints to agricultural productivity. PGPR play a crucial role in specifying plants resistance and adaptation to drought stress which could aid in solving future food security issues. Plant-PGPR interaction under drought stress alters the soil properties. PGPR bring out mechanisms such as triggering osmotic pressure and inducing of novel genes that play a crucial role in ensuring plant survival water deficit conditions. Approaches such as genetic engineering and plant breeding are essential in developing crops with drought tolerance potential, however inoculation of PGPR to ameliorate drought stress is a new application. Furthermore, future research is needed to address the identification of right kind of microbes and field evaluation of potential organisms.

Salinity stress in soil is mostly caused as an integrated result of anthropogenic environmental impact and climatic characteristics that limit natural vegetation processes, affecting soil and plant functioning. Mechanisms of PGPR in salt tolerance if acknowledged can contribute to a prolonged goal of interaction between plant and microbe so that we can exploit salinity affected regions for crop productivity. This can lead to counteract the negative effects on plants affected by salinity stress and avoiding and/or minimizing contaminants uptake. The results encourage future research on investigating their application in agriculture.

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Biogeographic distribution of chickpea rhizobia in the world

Junjie Zhang^{1,2}, Wenfeng Chen^{3,4}, Yimin Shang¹, Chen Guo¹, Shanshan Peng¹ and Wenxin Chen^{3,4}

¹College of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, P.R. China, ²Collaborative Innovation Center for Food Production and Safety of Henan Province, Zhengzhou, P.R. China, ³State Key Laboratory of Agrobiotechnology, Beijing, P.R. China, ⁴College of Biological Sciences and Rhizobium Research Center, China Agricultural University, Beijing, P.R. China

Chapter outline

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19.1 Introduction

Chickpea is a species from genus *Cicer* (Singh and Diwakar, 1995) and is an old world pulse and the second most important legume crop consumed worldwide (Ladizinsky, 1975; Iqbal et al., 2010). The name *Cicer* is of Latin origin, derived from the Greek word 'kikus' meaning force or strength (Singh and Diwakar, 1995). The word *arietinum* is also Latin, translated from the Greek 'krios', another name for both ram and chickpea (Singh and Diwakar, 1995). Chickpea also has a lot of different names such as garbanzo in Spanish, pois chiche in French, kichar or chicher in German, chana in Hindi, and gram or Bengal gram in English (Singh and Diwakar, 1995). chickpea is also with the name of 'nakhut' or 'nohut' in Turkey, Romania, Bulgaria, Afghanistan, and adjacent parts of Russia (Van der Maesen, 1987). Carbonized seeds of chickpea that have been unearthed in various archeological sites throughout the Middle East. The Middle East is also the homeland of several annual wild chickpeas, such as *C. judaicum* Boiss., *C. pinnatifidum* JAUB. et SPACH, and *C. bijugum* RECH which are apparently unrelated to the cultivated chickpea. Another two wild species of chickpea have been found in South-East Turkey, *C. echinospermum* DAVIS (DAVIS, 1969) and *C. reticulatum* LADIZ. (Ladizinsky, 1975).

It is well known that two distinct types of cultivated chickpea existed in the world, including Desi and Kabuli. Desi (*microsperma*) types were with pink flowers, anthocyanin pigmentation on stems with a colored and thick seed coat. While the kabuli (*macrosperma*) types were with white flowers, thin seed coat and smooth seed surface, but were without anthocyanin pigmentation on stem (Moreno and Cubero, 1978). In addition, an intermediate type with pea shaped seeds of local importance is recognized in India (Jukanti et al., 2012). The seed weight in desi and kabuli types were ranging from 0.1 to 0.3 g and 0.2 to 0.6 g respectively (Frimpong et al., 2010). The desi types account for about 80–85% of the total chickpea area and are mostly grown in Asia and Africa (Pande et al., 2005). The kabuli types are widely grown in the world, such as West Asia, North Africa, North America and Europe (Jukanti et al., 2012).

There are more than 50 countries growing chickpea including those from the Indian subcontinent, North Africa, the Middle East, southern Europe, the America and Australia (Jukanti et al., 2012). Globally, it is one of the most cultivated pulse in terms of world production, with 14.2 million tons of production and 0.96 tons ha⁻¹ for the average yield (FAO, 2014). As a cheaper source of protein, it is particularly important for poor countries in the world especially in developing countries lack of animal origins (Ramalho Ribeiro and Portugal Melo, 1990). Chickpea is usually cultivated

in regions where climate variability, drought and limited use of fertilizers significantly reduce productivity (Oliveira et al., 2017). India is the largest chickpea producing country, while other major chickpea producing countries include Pakistan, Turkey, Australia, Myanmar, Ethiopia, Iran, Mexico, Canada and USA (Jukanti et al., 2012). China is another chickpea producing country from Asia and the main planting area is from Xinjiang Province, Northwest China (Zhang et al., 2018b).

19.2 Diversity, geographic distribution and natural succession of chickpea rhizobia

During the past two decades, rhizobial diversity associated with chickpea has been studied in several countries, in Europe (Nour et al., 1994; Nour et al., 1995), Asia (Zhang et al., 2012b), and Oceania (Elias and Herridge, 2014). To date, *Mesorhizobium ciceri* (Nour et al., 1994), *M. mediterraneum* (Nour et al., 1995), *M. muleiense* (Zhang et al., 2012a) and *M. wenxiniae* (Zhang et al., 2018a) have been described to accommodate the rhizobial strains originally isolated from chickpea root nodules. In addition, several *Mesorhizobium* species, i.e., *M. amorphae*, *M. loti*, *M. tianshanense*, *M. oportunistum*, *M. abyssinicae* and *M. shonense* (Laranjo et al., 2004; Alexandre et al., 2009; Tena et al., 2017) were reported as additional potential symbionts of chickpea after lateral gene transfer acquisition of symbiovar *ciceri* gene set.

M. ciceri and *M. mediterraneum* are ubiquitous in most of the countries, Spanish, Portugal, Morocco, Tunisia and India, investigated in the previous studies (Fig. 19.1); however, none of them were found in China except *M. muleiense* and *M. wenxiniae*, documented in our previous studies in Xinjiang and Gansu Provinces Northwest China (Zhang et al., 2012a; Zhang et al., 2012b; Zhang et al., 2017; Zhang et al., 2018a). The geographic distribution of the chickpea species suggested that during the introduction of chickpea from one region to another, the seeds might contain some chickpea rhizobia during the harvest or it had been inoculated with chickpea rhizobia after introduction. In China, none of the popular chickpea rhizobia in the world has been found which indicated that the seeds were introduced for so long time the indigenous rhizobia had got the symbiotic ability with chickpea during the evolution.

For natural evolution research of chickpea rhizobia, an important work has been performed by Zhang et al. (2018b). The chickpea rhizobial species of *Mesorhizobium muleiense* from Xinjiang Province, Northwest China, was selected as it was the sole species associated with chickpea in that region. The *recA* genotypes were analyzed during the past decade, 2009–2016 and 28 genotypes were totally found of the species which indicated the natural succession of *M. muleiense*. Over sampling years and sites, four main genotypes were stable during the past years, while some genotypes were only found in the specific year or site which suggested a time-geographic succession model of the species.

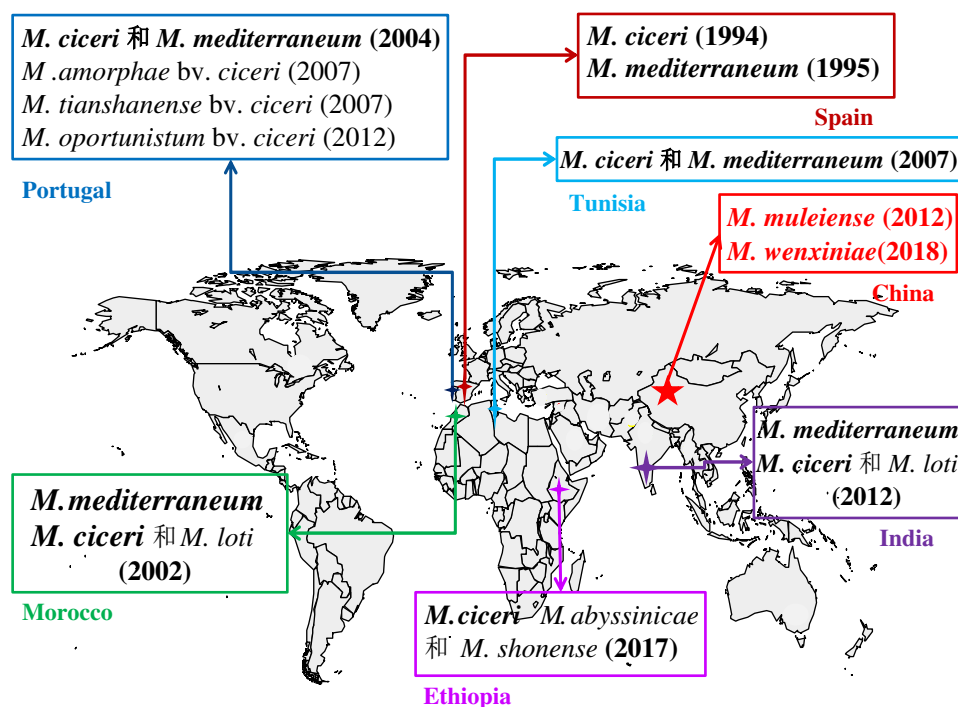


FIGURE 19.1 The distribution of chickpea rhizobial species found from the world.

Furthermore, though the four main genotypes were always found, the quantitative advantages of the genotypes were always different from different sampling years which might be caused from the management of the farms from human beings.

19.3 Effects of chickpea rhizobial inoculation on chickpea production

Soil is the habitat for prodigious number of living organisms which include the living forms both visible to the naked eye and on the other hand ones with microscopic dimensions (Singh and Singh, 2018). Among these, bacteria, especially rhizobia play a vital role in various biochemical processes taking place in the soil and are very much beneficial as far as the plant growth and soil properties are concerned (Anonymous, 2014). Among all, the N_2 fixing micro-organisms, symbiotic relationships between legumes and rhizobia are responsible for the largest contributions of fixed N to farming systems (Unkovich et al., 2008). Furthermore, it has been reported that rhizobial strains promote plant growth through other mechanisms such as phosphate solubilization ability in some legumes (Sridevi and Mallaiah, 2009) and lowering of plant ethylene levels due to presence of ACC deaminase activity (Glick, 2012).

Till date, nitrogen (N) fertilizers are crucial part of crop production and rank first among the external inputs to maximize agricultural production. However, N fertilizers contribute significantly to environmental pollution and have capability to disrupt every form of life to a great extent (Verma et al., 2014). Thus, the task of developing new production techniques which are sustainable both agronomically and economically should be paid more attention in the future. Biological nitrogen fixation (BNF) carried out by rhizobia-legume symbiosis can act as a renewable and environmentally sustainable source of N and can complement fertilizer inputs in crop production. It accounts for 65% of N currently used in agriculture (Gopalakrishnan et al., 2015). Under favorable conditions, the biological N_2 fixation process can make as much as 176 kg N per ha which could provide about 85% of the N requirements of legumes (Singh and Singh, 2018).

19.4 Effect of rhizobial inoculation on plant growth

Nitrogen fixers benefit the plant by providing them atmospheric nitrogen, which contributes to the development of plant growth and biomass production. Rudresh et al. (2005) studied the effect of inoculation with *Rhizobium* on growth attributes and observed that chickpea gave higher plant height (3.3%), number of branches per plant (23.3%) and biomass per plant (144%) as compared to uninoculated control. In similar findings, Elkoca et al. (2008) revealed that *Rhizobium* inoculation increased plant height, shoot dry weight and chlorophyll content in chickpea. These findings are in agreement with that of Giri and Joshi (2010) in chickpea. The increment in the root length was also observed in the inoculated treatments (Solaiman et al., 2011) which in return resulted in increased root surface area. The increase in root surface area enhances the nutrient acquisition by plant from the soil (Yadav and Verma, 2014). Branches are subsidiary parameter of plant biometry which are important to realize higher grain yields. Singh et al. (2011) found that number of primary and secondary branches were higher when chickpea was inoculated with *Rhizobium*. Similarly, Shahzad et al. (2014) reported greater plant height and shoot biomass per plant in *Rhizobium* inoculated chickpea in both irrigated and rainfed conditions. Beneficial effects on various growth attributes due to *Rhizobium* inoculation have also been reported (Singh et al., 2015) which may be attributed to enhanced growth and development of photosynthetic organs and the rate of photosynthates accumulation (Moinuddin et al., 2014) as well as production of various phytohormones such as indole acetic acid (IAA) (Verma et al., 2013).

19.5 Conclusions

The information presented here shows the origin and distribution of chickpea. It is the second most important legume crop in the world. It can be nodulated with diverse rhizobial species and form root nodules to fix nitrogen. *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* were the most prevalent species in many places of the world while they were not found in China where only *Mesorhizobium muleiense* and *Mesorhizobium wenxiniae* were reported. It indicated a clear geographic distribution of the chickpea rhizobia. In addition, it is evident from the studies that *Rhizobium* inoculation has positive impact on the growth, symbiotic parameters, yield and yield attributes in chickpea. For reducing the cost of cultivation there is a need of integrated use of *Rhizobium* and inorganic fertilizers. Especially during the introduction of chickpea to some regions where it has never been planted, rhizobial inoculation is necessary as it is always lack of indigenous chickpea rhizobia in the local soil. And the inoculation of highly efficient rhizobia will improve the quality, nutrition, yield and food safety of chickpea, as chickpea-rhizobia group could perform very

strong bio-nitrogen fixation and bio-growth promotion procedures and considerably decrease chemical nitrogen and pesticides and increase farmers' income.

In all, there is no doubt that chickpea is a very important legume pulse and is widely distributed in the world. The rhizobia inoculation during planting of chickpea will be helpful for chickpea, consumers and finally for food safety.

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Arbuscular mycorrhiza, a fungal perspective

Jelle van Creijl, Peng Wang and Erik Limpens

Laboratory of Molecular Biology, Wageningen University & Research, Wageningen, The Netherlands

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20.1 Introduction

Arbuscular mycorrhizal (AM) fungi are arguably the most successful endosymbionts of plants, forming an integral part of the life of most land plants under natural conditions (Brundrett and Tedersoo, 2018). They offer a range of benefits to their plant partners such as improved access to scarce nutrients in the soil (especially phosphate and nitrate), enhanced water uptake and water-use efficiency, resistance to abiotic (drought, salinity), protection from pathogens and herbivores, and general soil improvement including effects on the soil microbiome. This symbiosis originated >400 million years ago and has since been maintained in the majority of land plants. It is therefore of major ecological importance and potential for sustainable agriculture (Rodriguez and Sanders, 2015; Strullu-Derrien et al., 2018).

AM fungi are soil fungi belonging to the Glomeromycotina, a subphylum of Mucoromycota (Spatafora et al., 2016). Currently, ~300 AM fungal species have been described based on morphological characteristics, although molecular data suggest that this number may be vastly underestimated (Öpik and Davison, 2016). AM fungi are globally distributed and in general there is very little host-specificity in their interaction with plants, although host preferences may exist (Savary et al., 2018).

Most insights into the molecular aspects of AM symbiosis to date have been obtained from the plant side of this interaction. Particularly, molecular genetic studies in the model legumes *Medicago truncatula* and *Lotus japonicus* have provided a wealth of information on the plant components controlling this key symbiosis. Compared to this, insight into the fungal aspects has largely fallen behind. This was mostly caused by the lack of genomic information of these fungi and the inability to genetically manipulate them. In recent years however, major progress in this direction has been made, with the first elucidations of an AM genome sequence from *Rhizophagus irregularis* strain DAOM197198 (Tisserant et al., 2013; Lin et al., 2014). Currently, genome assemblies are available for 6 isolates of *R. irregularis* as well as for the AM species *R. clarus*, *R. diaphanus*, *R. cerebriforme* (Glomerales) and *Diversispora epigaea* (*Glomus versiforme*) and *Gigaspora rosea* (Diversisporales) (Ropars et al., 2016; Chen et al., 2018a,b; Maeda et al., 2018; Kobayashi et al., 2018; Sun et al., 2019; Morin et al., 2019).

These genome data have started to provide insight into the molecular components governing AM biology. In this chapter, we will highlight and address several of these recent molecular insights, specifically from the model AM fungus *R. irregularis*, such as communication with the plant host, (reciprocal) nutrient exchange, and the genetic make-up of AM fungi.

20.2 The *R. irregularis* life cycle

AM fungi are obligate biotrophs that need a living plant to be able to fulfill their life cycle. They are globally present in the soil both as spores and vast mycelium networks (Fig. 20.1A). Although AM fungal spores can germinate in the absence of a plant host, hyphal growth is only limited and rapidly stops if the fungus does not sense the presence of a plant. Upon the perception of plant-derived signals, the fungal hyphae show enhanced branching and growth, thereby enhancing their chance to reach a plant root. After contact, the fungus forms a hyphopodium on atrichoblasts of the root epidermis from which it penetrates the root. In response to signal exchange (see below), the plant cell reorganizes its cytoskeleton to form a specialized secretory structure, called the pre-penetration apparatus (PPA), that facilitates the entry of the fungus into the cell (Genre et al., 2011). Genome annotation has shown that AM fungi lack most cell wall-degrading enzymes (Tisserant et al., 2013; Morin et al., 2019). Therefore, they rely on the plant to weaken and break down the cell wall, after which they become enveloped by the plant plasma membrane. When the fungal hyphae reach the root cortex, they mainly grow in between the cells (Arum-type colonization), and/or spread in a cell-to-cell manner

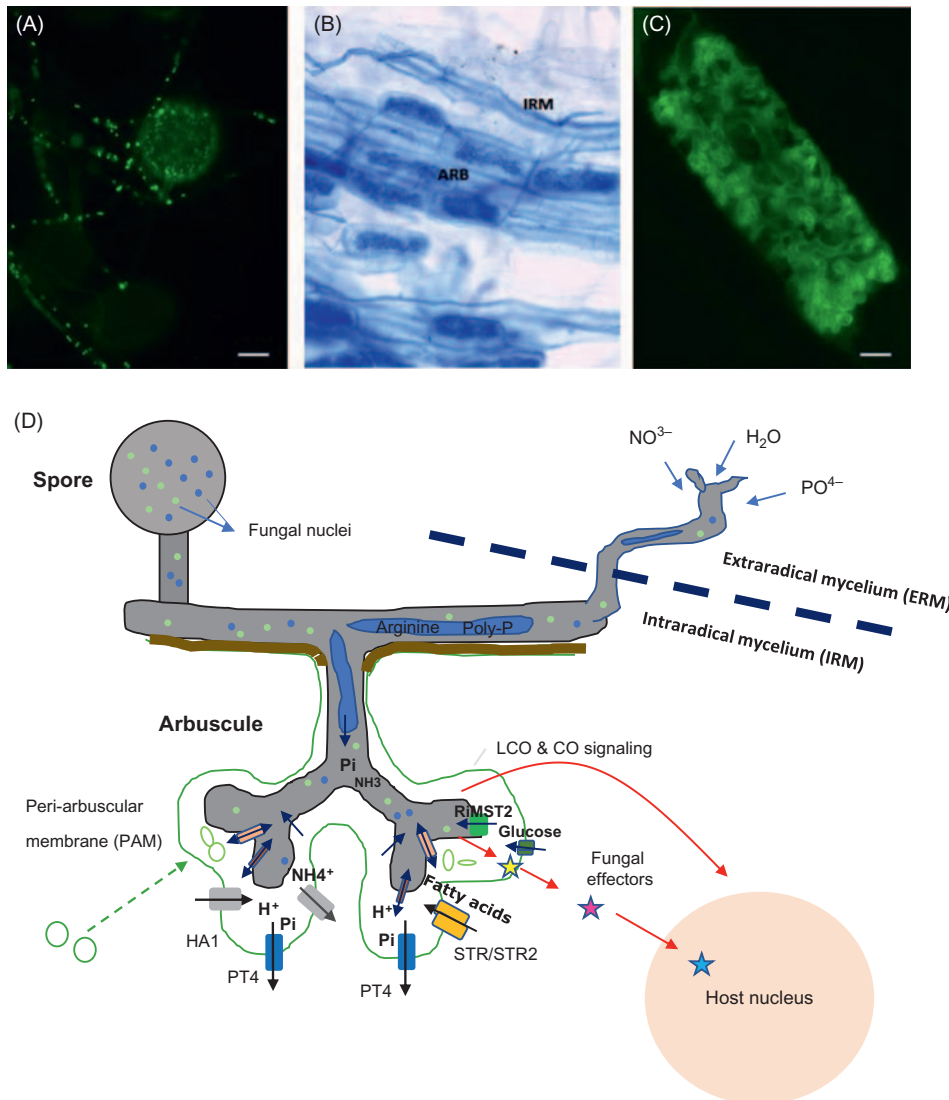


FIGURE 20.1 Arbuscular mycorrhizal lifestyle. (A) *Rhizophagus irregularis* spores and mycelium stained with SytoxGreen to mark the nuclei. A single spore typically contains hundreds of nuclei and the coenocytic mycelium forms a shared cytoplasm in which millions of nuclei migrate. (B) Trypan blue stained *Medicago truncatula* root colonized by *R. irregularis*. IRM = intraradical mycelium, i.e. fungal hyphae growing mostly in between cortical cells. ARB = arbuscules. (C) Confocal image of an Arbuscule where the peri-arbuscular membrane is marked with a green-fluorescent plasma membrane marker. (D) Schematic drawing of various aspects of Arbuscular mycorrhiza, for details see main text.

(Paris-type) depending on the plant species (Kubota et al., 2005). Ultimately, they reach the inner cortical cells where they enter and form highly branched hyphal structures, called arbuscules (Fig. 20.1B and C). There they become surrounded by a specialized plant membrane (the peri-arbuscular membrane) creating a symbiotic interface where primary exchange of nutrients takes place (Gutjahr and Parniske, 2013; Harrison and Ivanov, 2017). Recently, detailed electron microscopy combined with tomography showed that the interface is more complex than previously thought. Extensive tubular and vesicular extracellular membranes, either secreted as vesicles or extrusions of the plasma membranes, were observed in the peri-arbuscular space. Furthermore, extensive, interconnected tubular fungal plasma membrane extensions were observed in the paramural space of arbuscules and intraradical hyphae (Ivanov et al., 2019; Roth et al., 2019). Such paramural structures are not restricted to AM fungi, as similar structures have been observed in a wide range of fungi, where they are called lomasomes or plasmalemmasomes and thought to play a role in nutrient absorption. Extracellular vesicles have also been associated with defense responses (Limpens, 2019).

Arbuscules are relatively short lived structures that degenerate after a couple of days. It is currently unknown why arbuscules degenerate or which of the two partners initiates their degeneration. It has been shown that the life-time of arbuscules can be controlled by the host plant (Kobae et al., 2010, 2014; Floss et al., 2017), which likely gives the plant a means to prevent the interaction from becoming parasitic if the fungus does not provide sufficient nutrients. This hypothesis is supported by the observation that a lack of phosphate delivery, due to the mutation of an arbuscule-specific plant phosphate transporter or a proton pump that fuels it, leads to the premature collapse of arbuscules (Krajinski et al., 2014; Wang et al., 2014; Fig. 20.1D).

After the first establishment of arbuscules, fungal hyphae continue to spread through the root and form (lipid-filled) storage structures called vesicles. Colonization of the root continuously occurs via intraradical mycelium (IRM) that go on to make new arbuscules, as well as extraradical mycelium (ERM) and spores that establish new infection sites. As the fungus grows it can be in contact with multiple host plant species at the same time. Similarly, a plant is often colonized by several different AM fungi (Berruti et al., 2013), creating an underground network through which plants are interconnected and nutrients and signals can be exchanged (van der Heijden et al., 2015). Generally, the hyphal network forms a continuous cytoplasm through which millions of nuclei migrate, although the fungus can make septa to seal off stressed or damaged parts of the hypha. It was shown that nuclei fill up the spores by migrating from the hypha into the developing spores as they are being formed (Marleau et al., 2011). As a result, the spore typically contains several hundreds of nuclei, and there is never a single nucleus stage that generates the next generation (Sanders and Croll, 2010; Fig. 20.1A). Genetically related AM fungi have been shown to be able to undergo anastomosis (hyphal fusion) and exchange nuclei, however a sexual stage or conclusive evidence for meiosis has not yet been found. The current ideas on the genetic make-up of AM fungi are discussed further on in this chapter.

20.3 Communication with the host plant

Communication between both partners starts before the fungus contacts the plant root through the exchange of signal molecules. The plant root secretes among others flavonoids, 2-hydroxy fatty acids and strigolactones, that are perceived by the fungus (Bécard et al., 1995; Nagahashi and Douds, 2011; Nadal and Paszkowski, 2013). Best studied is the role of strigolactones, which stimulate the germination and branching of hyphae and trigger the activation of mitochondria and metabolism in the fungus (Akiyama et al., 2005; Besserer et al., 2006, 2008). In addition to their role as symbiotic signals perceived by AM fungi, strigolactones are also endogenous plant hormones that control plant architecture. Besides strigolactones, the plant hormones ethylene and cytokinin may also be perceived by AM fungi. Ethylene was shown to promote AM spore germination and hyphal growth and homologs of plant ethylene and cytokinin receptors have been identified in the genome of *R. irregularis* (Herivaux et al., 2017). However, the involvement of these receptors in plant hormone perception still needs to be assessed.

An N-acetylglucosamine transporter called NOPE1 has been identified in rice and maize and was shown to be required for the induction of symbiotic transcriptome changes in the fungus by root exudates (Nadal et al., 2017). NGT1, a homolog of this transporter in *Candida albicans*, is required for the uptake of N-acetylglucosamine to control proliferation, signaling, and virulence (Alvarez and Konopka, 2007). Therefore, it is hypothesized that NOPE1 may transport a still-to-be-identified molecule that is perceived by AM fungi to prime them for symbiosis (Nadal et al., 2017).

20.3.1 (Lipo-)chitoooligosaccharides

In turn, AM fungi secrete diffusible molecules that trigger responses in the plant, such as the induction of calcium oscillations in and around the nucleus (known as calcium spiking), stimulation of lateral root formation, activation of

symbiosis-specific genes, and formation of the PPA. The best studied AM signals are chitin-like lipo-chitoooligosaccharides (myc-LCOs) and short chain chitoooligosaccharides (COs), together called myc-factors (Maillet et al., 2011; Genre et al., 2013). LCOs consisting of β -1-4-linked N-acetyl glucosamine residues with an acyl chain at the non-reducing residue were first identified in rhizobium bacteria. These are called Nod factors and are essential to initiate a nitrogen-fixing endosymbiosis with legume plants. Several decades of research have led to the identification of a plant signaling pathway that is activated by Nod factors and required for both the rhizobium as well as AM symbiosis. Therefore, this pathway has been called the common symbiotic (SYM) signaling pathway (Parniske, 2008; Oldroyd, 2013). In line with its essential role in establishing an AM symbiosis, the common SYM pathway is conserved in all AM host plants and has typically been lost in non-host plants such as *Arabidopsis thaliana* (Delaux et al., 2014; Bravo et al., 2016).

R. irregularis produces a mix of sulfated and non-sulfated tetrameric and pentameric LCOs mostly acylated with either an oleic acid (C18:1) or palmitic acid (C16:0) (Maillet et al., 2011). Although various genes encoding chitin synthases and deacetylases are present in the fungal genome, it is currently unknown how lipid-modified LCOs are made. Direct homologs of the rhizobial *nod* genes, such as *nodC* which encodes an enzyme that attaches the fatty acyl chain to the GlcNAc backbone, have not been found in the fungal genome.

In addition to LCOs, short-chain chito-oligosaccharides (COs) lacking an acyl chain have been proposed to play a role in AM symbiosis (Genre et al., 2013). Tetrameric and pentameric COs were identified in germinated spore exudates at markedly higher concentrations than myc-LCOs, and their secretion was induced by the application of strigolactones (Genre et al., 2013). Such short-chain COs are also able to trigger calcium oscillations in epidermal cells of legume as well as in non-legume roots. Like myc-LCO signaling, CO-induced signaling is dependent on the SYM pathway.

Myc-LCOs can stimulate mycorrhization in a variety of plant species at sub-nanomolar concentrations, indicating that they are efficiently perceived by a broad range of plants. Myc-LCOs are thought to be perceived by LysM domain containing receptor-like kinases (LysM-RLK), in analogy to the perception of rhizobial Nod factors. In the model legumes *M. truncatula* and *L. japonicus*, Nod factors are perceived by a complex of a LysM-domain containing receptor containing a functional kinase domain (MtLYK3/LjNFR1) and one with a non-functional kinase domain (MtNFP/LjNFR5). Although these receptors have been implicated in AM symbiosis (Maillet et al., 2011; Czaja et al., 2012; Zhang et al., 2014a,b), knock-out mutants are still able to establish an AM interaction. This suggests that additional (redundant) receptors are involved or that additional fungal signals are able to activate the SYM pathway. In rice, a single LysM-RLK, called OsCERK1, was shown to be essential for establishing an AM interaction (Miyata et al., 2014; Zhang et al., 2014a,b). Interestingly, OsCERK1, like its ortholog Arabidopsis CERK1, is also required for chitin-triggered immune responses. This raises the question how a plant distinguishes between beneficial AM fungi and fungal pathogens (van Zeijl et al., 2015; Zipfel and Oldroyd, 2017). OsCERK1 likely forms a complex with an NFP/NFR5-type LysM receptor and it is the specificity of the complex that may determine whether symbiotic responses or defense is activated. In line with this, it was recently shown in *M. truncatula* and *L. japonicus* that paralogues of the Nod factor receptors, MtLYK9/MtLYR4 and LjLYS6, are required for chitin/chito-oligosaccharide-triggered immune responses (Bozsoki et al., 2017). This indicates that the duplication and subsequent divergence of LysM-RLKs led to the emergence of distinct roles in symbiosis (De Mita et al., 2014; Gough et al., 2018). Double mutants of MtNFP and MtLYR4 show a much more drastic reduction in mycorrhization (Giles Oldroyd, personal communication), which suggests that both myc-LCOs and short-chain COs are used symbiotic signals. Additionally, AM fungi can actively suppress the chitin-triggered immune response through the secretion of effector proteins (see below).

20.3.2 Effectors

Another class of signal molecules released by AM fungi are (small) secreted proteins that act as potential effector proteins. In plant-pathogen interactions ‘effectors’ are defined as pathogen-secreted proteins that modulate host plant defense response and enable colonization of plant tissue (Hogenhout et al., 2009).

In nature, plants are constantly exposed to multiple micro-organisms, ranging from pathogenic and commensalistic to symbiotic. The cell wall of AM fungi contains several molecules, such as chitin and β -glucans, that are well characterized general fungal molecule-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), which induce host defense responses called PAMP-triggered immunity (Lo Presti et al., 2015; Fesel and Zuccaro, 2016; Rovenich et al., 2016). This raises the question how AM fungi manage to intracellularly colonize such an extremely broad range of plants (> 80% of land plants).

The first evidence that AM fungi secrete effector proteins to manipulate the immune system of the host was provided in 2011, when it was shown that *R. irregularis* secretes a protein, called Secreted Protein 7 (SP7), that was able

to translocate to the plant cell nucleus (Kloppholz et al., 2011). It was shown to interact with a pathogenesis-related transcription factor, ERF19, in *M. truncatula* roots, and could interfere with ERF19-dependent defense gene induction. In line with this, expression of RiSP7 in the hemibiotrophic fungal rice pathogen *Magnaporthe oryzae* was able to increase the biotrophic phase of this interaction. Cell- and stage-specific transcriptome analyses have shown that SP7 is mainly expressed in the extracellular hyphae in close contact with the root, but not or hardly when the fungus grows inside the root (Zeng et al., 2018). This suggests that it mainly plays a role at the first contact at the epidermis. The occurrence of KEX2 protease cleavage sites in SP7 have led to the suggestion that it may in fact be cleaved before secretion, raising the hypothesis that the resulting peptides may be biologically active (Kamel et al., 2017), however evidence is currently lacking. Intriguingly, follow-up work on the role of SP7 in AM symbiosis has suggested that SP7 may affect the phosphate starvation response in plants (Natalia Requena, personal communication). It will be interesting to see whether this would be a strategy used by the fungus to make the plant more reliant on the fungus to obtain phosphate.

With the release of the first *R. irregularis* genome sequence, the number of potential effector candidates was increased significantly, with >600 predicted secreted proteins (SPs) (Tisserant et al., 2013; Lin et al., 2014; Toro and Brachmann, 2016; Kamel et al., 2017; Zeng et al., 2018). It should be noted that the number of SPs varies between studies because the genome assemblies (gene models) and prediction pipelines that were used differ. A detailed analysis of the stage- and host species-dependent expression profile of secreted *R. irregularis* proteins was performed by Zeng et al. (2018). Laser microdissection combined with RNA-seq was used to monitor the expression of SP encoding genes in germinating spores, ERM, IRM, and arbuscules. Furthermore, expression was analyzed in three different host plants (*Allium schoenoprasum*, *M. truncatula* and *Nicotiana benthamiana*) to determine whether the fungus uses a distinct set of effectors in the interaction with different host plants. This work revealed the expression of ~300 RiSPs that could be reliably confirmed by RNA-seq data. Several of these SPs were predicted to be targeted to the nucleus, suggesting that they may be translocated to the host nucleus similarly to SP7. The majority (85%) of the SPs was more or less equally expressed in the interaction with all three plant hosts, however a subset was expressed in a host-dependent manner. A similar observation of putative host-dependent expression of effectors was made by Kamel et al. (2017), who monitored SP expression in *M. truncatula*, *Brachypodium distachyon* and the liverwort *Lunularia cruciata*. Host-dependent expression of such putative effectors raises the hypothesis that they may play a role in controlling symbiotic efficiency and adaptability to different hosts. A comparison of the genome sequences of 6 different *R. irregularis* isolates and 5 AM species further indicated substantial variation in the repertoire of candidate effector proteins (Chen et al., 2018a,b; Morin et al., 2019).

Unraveling the function of the putative effectors in AM symbiosis is a major challenge. This is especially hampered by the fact that AM fungi currently cannot be stably transformed (Helber and Requena, 2008). As an alternative, host-induced gene silencing (HIGS; Nowara et al., 2010) has been successfully used in some studies to silence AM fungal genes, although the efficiency of this method appears to vary significantly. Using this method, to date three additional effectors have been identified that influence AM symbiosis.

Tsuzuki et al. (2016) searched for effector proteins that are up-regulated in germinating spores in response to strigolactone perception. This identified 11 putative effectors among which strigolactone-induced putative secreted protein 1 (SIS1). HIGS of SIS1 in *Agrobacterium rhizogenes* transformed root cultures of *M. truncatula* resulted in suppression of colonization and the formation of stunted arbuscules. A role in efficient intraradical colonization fits with its high expression in IRM as well as in arbuscules (Zeng et al., 2018). The molecular mechanism by which SIS1 controls fungal colonization is currently not known.

Another effector from *R. irregularis* that showed induction upon strigolactone perception consisted of a signal peptide followed by a single LysM domain, and was named secreted LysM protein (RiSLM) (Schmitz et al., 2019; Zeng et al., 2019 unpublished). Stage-specific expression analyses showed that it is most highly expressed in the IRM. LysM domain containing effectors have mostly been studied in pathogenic fungi, where they were shown to bind chitin-oligosaccharides (Sánchez-Vallet et al., 2015). Such effectors, containing one to three LysM domains, were reported to protect fungal hyphae from plant chitinases and/or to interfere with chitin-triggered immunity. Purification of RiSLM in combination with binding studies confirmed that it can also bind both long-(CO7/8) and short-chain COs (CO4) and that it also binds both sulphated and non-sulphated myc-LCOs (Zeng et al., 2019). Furthermore, bioassays showed that it can both protect fungal hyphae against hydrolysis by plant chitinases and interfere with chitin-triggered immunity, analogous to pathogenic LysM effectors. HIGS of RiSLM in *M. truncatula* hairy roots caused a significant reduction in fungal colonization (Zeng et al., 2019 unpublished). This indicates that LysM effectors are a common strategy used by both symbiotic and pathogenic fungi to colonize plants. Despite its ability to bind short-chain COs or myc-LCOs, co-application of RiSLM did not significantly block the induction of symbiotic gene expression. This is likely caused

by a higher affinity of the symbiotic receptor complexes for myc-LCOs. The affinity of RiSLM for COs is in the same range as reported for chitin immune-receptors, raising the question how it can efficiently interfere with chitin-triggered immunity. A comparison of RiSLM homologs in different AM species and *R. irregularis* isolates showed a strikingly high level of variation in the protein sequence and indications of positive selection (Schmitz et al., 2019; Zeng et al., 2019). Besides LysM effectors, Crinkler (CRN) effectors represent a well-known class of effectors that was first identified in pathogenic oomycetes and is also found in AM fungi (Lin et al., 2014; Voss et al., 2018). These effectors are characterized by a highly conserved N-terminal LFLAK domain, consisting of a highly conserved LxLFLAK motif and a neighboring DWL domain (Torto et al., 2003). The LFLAK domain is required to translocate CRNs into the plant cell where they exert their function in the host nucleus (Schornack et al., 2010). Different functions have been reported for CRNs, which may be derived from the various C-terminal domains, ranging from induction of cell death to protection from biotic and abiotic stresses (Torto et al., 2003; Liu et al., 2011; Rajput et al., 2015; Zhang et al., 2015). In *R. irregularis* at least 42 putative CRN homologs were identified (Lin et al., 2014; Voss et al., 2018). Of these only a small set contains a signal peptide. Similarly, in oomycetes only a small subset of CRNs contain a signal peptide, and the ones without one are believed to be subject to alternative secretion pathways (Haas et al., 2009; Gaulin et al., 2018). One of the *R. irregularis* CRNs containing a signal peptide, RiCRN1, was shown to be significantly more highly expressed in IRM and arbuscules. It was shown to form homodimers and to localize to nuclear bodies suggesting that, similarly to other CRNs, it functions in the plant nucleus (Voss et al., 2018). Suppression of RiCRN1 expression via HIGS caused a reduction of root colonization. Surprisingly, ectopic expression of RiCRN1 led to a drastic reduction in arbuscule number and size, indicating a role in arbuscule formation and development. Therefore, it was suggested that a tight regulation of RiCRN1 expression is critical for proper arbuscule development. Clearly, many more exciting insights into the role of effector proteins in AM symbiosis are expected to be revealed in the future.

20.4 Nutrient exchange

AM fungi are obligate biotrophs that need a living plant to full-fill their life cycle. By providing mineral nutrients to their host they obtain fixed carbon in return. This exchange of nutrients has been compared to a market setting, where reciprocal rewarding between both partners is thought to stabilize the symbiosis (Kiers et al., 2011). In *M. truncatula* it was shown that a fungus that is less cooperative, i.e. provides less phosphate, gets less carbon from the plant than a more cooperative fungus. In turn, when the plant gives less carbon to the fungus, the fungus provides less phosphate to the plant (Kiers et al., 2011; Fellbaum et al., 2014). Modeling approaches have suggested that reciprocal exchange may be the result of the two partners competing for the same nutrient sources (Schott et al., 2016). Subsequent modeling provided a basic thermodynamic model that may explain the underlying market forces, with a key role for proton pumps to fuel the nutrient exchanges (Dreyer et al., 2019). How this reciprocal rewarding works in (complex) natural settings is a major question in AM ecology (Walder and van der Heijden, 2015). A study where a shared AM hyphal network was established between flax and sorghum showed that depending on the AM fungal species, the plant that provided most carbon to the network did not obtain the most phosphate/nitrogen in return (Walder et al., 2012). A proper understanding of the molecular components controlling nutrient exchange in both plant and fungus as well as colonization levels will therefore be required to better understand this key aspect of the symbiosis.

20.5 From fungus to plant

20.5.1 Phosphate

Fungal hyphae form a vast and dense mycelial network and are much better able to explore the soil for scarce mineral sources than the relatively large plant roots. Plants can only take up soluble inorganic orthophosphate (Pi), but most phosphate in the soil is present in bound forms, with large parts as organic P (i.e. phytate). Extraradical hyphae of AM fungi have been shown to exhibit both phytase and acid phosphatase activity to mineralize organic P (Wang et al., 2017a,b). Although acid phosphatases are expressed in the extraradical hyphae, phytase-encoding genes have not been reported in AM fungi. It is thought that fungal hyphae-associated bacteria are responsible for the enhanced phytase-activity. Indeed, it was shown that AM hyphal exudates can promote the growth of phytase-producing bacteria (Zhang et al., 2014a,b, 2016).

Mineralized Pi is subsequently taken-up by the fungus through the activity of Pi transporters. Proton(H⁺/Pi)- and sodium(Na⁺/Pi)-coupled Pi transporters have been identified in AM fungi (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001; Tisserant et al., 2012, 2013; Fiorilli et al., 2013). The first AM H⁺/Pi transporter was

identified in *G. versiforme* and it was shown to be primarily expressed in extraradical hyphae (Harrison and van Buuren, 1995). Corresponding H^+ -ATPases, providing the force to drive the uptake of Pi across the extraradical hyphal membrane, have been subsequently identified (Ferrol et al., 2000; Requena et al., 2003). Many of the fungal Pi transporters are also expressed in the arbuscules. This may indicate a competition between plant and fungus at the interface to control the amount of Pi delivered, or a role in phosphate secretion from the arbuscules (Xie et al., 2016; Ferrol et al., 2019). The functional involvement of a high-affinity fungal Pi transporter, expressed throughout the IRM and ERM, was only recently revealed in *Gi. margarita* (Xie et al., 2016). HIGS of phosphate transporter *GigmPT* led to reduced colonization and premature collapse of arbuscules. Interestingly, the same study revealed that *GigmPT* can function both as Pi transporter as well as Pi sensor, forming a transceptor in analogy to the phosphate transceptor Pho84 in yeast. Silencing of *GigmPT* affected PHO- and PKA-dependent Pi-signaling. Furthermore, it was shown that increased carbon availability induced the expression of *GigmPT* in extraradical hyphae under low phosphate conditions, corresponding with increased P transfer to the host.

Once taken up, Pi is polymerized into polyphosphate (poly-P) in the vacuoles (Ezawa et al., 2002; Fig. 20.1D). This likely occurs by the vacuolar transporter chaperon complex (VTC), which controls poly-P synthesis in yeast. Several VTC components are upregulated when Pi is applied to extraradical AM hyphae (Kikuchi et al., 2014). The poly-P is then transported to the intraradical hyphae via the mobile vacuolar system and cytoplasmic streaming (Uetake et al., 2002). Recently it was shown that water transport across the plasma membrane in the intraradical hyphae by the aquaporin *RcAQP3* of *R. clarus* influences poly-P transport (Kikuchi et al., 2014). Virus-induced silencing of *RcAQP3* as well as the reduction of host transpiration reduced the rate of poly-P translocation from extraradical to intraradical hyphae. This suggests that host transpiration drives water transport through the hyphae.

A major question that remains is how phosphate is released by the fungi to be taken up by the host plant at the arbuscules. It is thought that poly-P is hydrolyzed inside by the fungus inside the root (Kikuchi et al., 2014), after which it is transported across the vacuolar membrane, possibly by the vacuolar Pi transporter PHO91 (Tisserant et al., 2012). Subsequently the Pi is transported across the fungal plasma membrane into the peri-arbuscular space from which it is taken up by specific phosphate transporters into the plant (Ferrol et al., 2019; Fig. 20.1D). The identity of the proposed candidate Pi efflux transporters however is still unknown. Relevant in this aspect, a H^+ -coupled Pi transporter HcPT2 from the ectomycorrhizal fungus *Hebeloma cylindrosporum* was shown to be involved in the export of Pi towards *Pinus pinaster* roots (Becquer et al., 2018; Dreyer et al., 2019). Alternatively, a role for a plasma membrane localized VTC complex and export of poly-P followed by hydrolysis by a plant acid phosphatase in the peri-arbuscular space has been proposed (Ezawa and Saito, 2018). Additionally, the exchange of vesicles may be involved in the transport of nutrients (Ivanov et al., 2019; Roth et al., 2019).

20.5.2 Nitrogen

Besides providing phosphate, AM fungi also play a key role in nitrogen (N) supply to host plants (Garcia et al., 2016; Wipf et al., 2019). The fungal ERM can take up inorganic (NH_4^+ or NO_3^-) or organic nitrogen (amino acids/short peptides). AM fungi contain a few high affinity nitrate transporters (NRTs; Tian et al., 2010; Koegel et al., 2015) as well as ammonium transporters (López-Pedrosa et al., 2006; Pérez-Tienda et al., 2011; Calabrese et al., 2016). Several of these transporters, such as RiNRT2, are expressed in both the ERM and IRM, suggesting that competition for nitrogen sources at the symbiotic interface may occur as was suggested for phosphate, or that bidirectional transfer can occur. Once taken up, nitrogen ions are converted via glutamine into arginine through the GS/GOGAT cycle (Wipf et al., 2019). Arginine is then transported to the IRM (Fig. 20.1D), where it is broken down into urea and ornithine (Govindarajulu et al., 2005; Jin et al., 2005). To be transported, the positively charged arginine may form complexes with the negatively charged poly-P granules. Hydrolysis of urea then generates ammonium which is released into the peri-arbuscular space to be taken up by the plant by specific ammonium transporters (Kobae et al., 2010; Breuillin-Sessoms et al., 2015). Interestingly, it was found that premature arbuscule degeneration in the arbuscule-specific *M. truncatula* phosphate transporter (PT4) mutant is suppressed when plants are starved for nitrogen. This suppression requires the ammonium transporter MtAMT2;3 (Breuillin-Sessoms et al., 2015). Because MtAMT2;3 failed to complement a yeast mutant impaired in ammonium transport, it was suggested that it may have an N-sensing and signaling function.

Organic nitrogen transporters, such as di- and tripeptide transporters (PTRs) or amino acid permease, such as GmosAAP1 from *Funnelformis mosseae* (Cappellazzo et al., 2008) have been found to be induced in AM fungi. One of the dipeptide transporters, RiPTR2 in *R. irregularis*, was shown to be more highly expressed in IRM compared to ERM, and to be expressed in arbuscules as well (Belmondo et al., 2014). Again, this finding suggests that the fungus

may competitively reabsorb nutrients released in the peri-arbuscular space and thus exert a control over the amount of nutrients delivered to the host.

20.5.3 Potassium transport

The third mineral, next to P and N, that is often most limiting plant growth under natural conditions is potassium (K). Several studies indicate also a positive effect of AM symbiosis on plant K acquisition (Garcia et al., 2016). Uptake of K from the soil may be dependent on the high affinity K uptake transporter (RiHAK) that has been identified in *R. irregularis* (Garcia et al., 2016). How the K is subsequently transferred to the host plant is not known.

20.5.4 Metals and other minerals

In addition to the main mineral nutrients phosphate, nitrate, and potassium, numerous additional transporters for metals and minerals such as sulfate can be identified in the *R. irregularis* genome (Tisserant et al., 2013; Garcia et al., 2016). With the availability of cell-specific transcriptome analyzes, putative transporters can now be mined to identify key candidates for a role at the arbuscule interface (Zeng et al., 2018). Furthermore, the role of these transporters in the symbiosis remains to be investigated.

20.6 From plant to fungus

20.6.1 Hexoses

It was long thought that sugars (hexoses) were the main carbon source provided by the plants across the peri-arbuscular membrane to the AM fungus (Roth and Paszkowski, 2017). This was mainly based on studies with isotope-labeled sugars added to mycorrhized roots in combination with detailed NMR analyzes. These indicated that glucose can be directly transferred from the host to the fungus (Shachar-Hill et al., 1995; Solaiman & Saito, 1997; Pfeffer et al., 1999; Bago et al., 2000; 2003). Furthermore, the ERM was shown to be largely unable to take up sugars. In agreement with a role for plant-derived hexoses, a fungal proton-coupled monosaccharide transporter, RiMST2 from *R. irregularis*, was shown to be required for the proper development of arbuscules and fungal colonization through HIGS (Helber et al., 2011; Fig. 20.1D). MST2 is most highly expressed in arbuscules but also showed expression in the IRM and ERM (Helber et al., 2011; Zeng et al., 2018). This led to the proposition that AM fungi may also take up sugars from the apoplast through their intraradical hyphae. Furthermore, cell wall derived xylose was shown to trigger the upregulation of *RiMST2* expression, suggesting that cell wall monosaccharides may also be taken up by AM fungi (Helber et al., 2011). Several MST members show preferential expression in the ERM, where they may play a role in the uptake of organic C (Roth and Paszkowski, 2017). At present, the plant sugar transporters that export hexoses across the peri-arbuscular membrane are unknown, however it is likely that SWEET (Sugars Will Eventually be Exported) transporters are involved as several SWEET members are induced during the AM symbiosis (Manck-Götzenberger and Requena, 2016; Kafle et al., 2019). A recent study in *M. truncatula* identified a single SWEET gene (MtSWEET1b) that is significantly expressed in arbuscule cells and localizes to the peri-arbuscular membrane (An et al., 2019). It was shown to transport glucose. Furthermore, expression of dominant-negative forms of this SWEET caused an early collapse of arbuscules. This suggests that glucose transport to the arbuscule plays a role in the maintenance of the interface, although a role for MtSWEET1b in transport of other molecules, such as gibberellic acid (Kanno et al., 2016) cannot be excluded. After uptake of hexoses from the plant, the sugars can be converted into glycogen as storage form and transported to the ERM. However, the absence of a gene encoding glucoamylase in the genome of AM fungi, suggest they may not be able to use glycogen effectively (Kobayashi et al., 2018).

20.6.2 Fatty acids

The view of hexoses as major sole carbon source for AM fungi recently changed when it was found that AM fungi lack a key enzyme, type-I multidomain fatty acid synthase (FAS), in their genome, required to efficiently synthesize longer chain (C16:0) fatty acids (Wewer et al., 2014; Chen et al., 2018a,b). This was striking as lipids are in fact the major carbon storage form in AM fungi (Rich et al., 2017; Wang et al., 2017a,b). It suggested that AM fungi are fatty acid auxotrophs that need to obtain fatty acid precursors from the plant (Fig. 20.1D). Correspondingly, the plant induces a whole set of genes involved in fatty acid biosynthesis specifically in the arbuscule-containing cells (Bravo et al., 2017; Jiang et al., 2017; Luginbuehl et al., 2017; Limpens and Geurts, 2018; An et al., 2018). Mutations in these genes typically

cause the premature collapse or underdevelopment of arbuscules and overall reductions in fungal colonization. By expressing a fatty acyl-ACP thioesterase (UcFatB) that produces lauric acid (C12:0), which is normally not or very lowly present in mycorrhizal roots or AM fungi, it was shown that indeed the plant provides fatty acids to the AM fungi (Jiang et al., 2017; Luginbuehl et al., 2017). The fatty acid precursor that is likely transported is β -monacylglycerol (β -MAG). Inside the fungus β -MAG is converted into the storage form triacylglycerol (TAG) to be transported to the ERM and stored in spores and vesicles. TAG can be converted back into sugars through the glyoxylate cycle and gluconeogenesis (Bago et al., 2003), and therefore fatty acids are likely a major nutritive carbon source for the fungus. Currently, the different contributions of sugars and fatty acids to feed the fungus remain to be addressed.

20.6.3 Micronutrients

Besides the inability to efficiently synthesize fatty acids, the available AM genome sequences further indicate that AM fungi are not able to synthesize essential micronutrients such as thiamine (vitamin B1) or vitamin B6 (Tisserant et al., 2013; Kobayashi et al., 2018; Morin et al., 2019). Therefore, these micronutrients are likely also provided by the plant host.

Intriguing in this respect is the report by Hildebrandt et al. (2002, 2006), who found that co-culturing of the AM fungus *R. irregularis* Sy167 with a mixture of two isolates of the bacterium *Paenibacillus validus* allowed the growth of the fungus even up to the formation of fertile spores. It was found that the *P. validus* isolates secrete raffinose which could stimulate the growth of fungal hyphae, but not the formation of spores. Given the fatty auxotrophy of AM fungi, it must mean that the *P. validus* cultures also contained fatty acids, and micronutrients, at sufficient levels to sustain the development of hyphae and spores.

20.7 Genetic make-up of AM fungi

AM fungi used to be classified with Mucoromycotina under the phylum Zygomycetes based on shared phenotypical traits (Spatafora et al., 2016; Orchard et al., 2017). However, molecular phylogenetic analyzes have shown that these groups form two separate subphyla. AM fungi are now considered to form a subphylum of the Mucoromycota, called Glomeromycotina (Spatafora et al., 2016), which share characteristics such as the development of a coenocytic mycelium and phylum-specific gene families (Lin et al., 2014; Morin et al., 2019). AM fungi diverged quite early (> 450 Mya) and have a striking genetic make-up that may be related to their early endosymbiosis with plants. The genomes of the six currently sequenced AM fungal species are among the largest genomes in the fungal kingdom, ranging from 126 to 598 Mb (Ropars et al., 2016; Chen et al., 2018a, b; Maeda et al., 2018; Kobayashi et al., 2018; Sun et al., 2019; Morin et al., 2019). These substantial differences are mainly due to the expansion of transposable elements (TEs, 30–60% of the genome). Furthermore, several gene families are strikingly expanded in the Glomeromycotina. Among these are for example proteins with a kinase domain, especially tyrosine kinases often associated with Sel1-repeats involved in protein-protein interactions (Mittl and Schneider-Brachert, 2007), and BTB domain (BTB-POZ and BTB-Kelch) containing proteins, a protein–protein interaction domain found in proteins involved in a wide range of cellular functions such as transcriptional regulation and chromatin remodeling, protein degradation and cytoskeletal regulation (Chaharbakhshi and Jemc, 2016). Characteristic for AM fungi is also the absence of most plant cell wall degrading genes, genes required for degradation of sucrose and glycogen (invertase, glucoinvertase, and glucoamylase), non-ribosomal peptides and genes required for the biosynthesis of polyketides, thiamine and fatty acids (Morin et al., 2019). These metabolic adaptations are likely causally related to their obligate biotrophy and intimate interaction with plants.

AM fungi propagate asexually by forming spores. A sexual cycle and related structures have never been observed in AM fungi. Therefore, they have been called “ancient asexual scandals” (Judson and Normark, 1996). This view has recently been challenged with the identification of all core meiotic genes in AM fungi, suggesting that they may have the capacity to undergo meiosis (Halary et al., 2011). In line with this, Ropars et al. (2016) identified a putative mating type locus in *R. irregularis* by sequencing 5 different isolates (Koch et al., 2004). This locus consists of two homeobox domains containing proteins that are structurally and evolutionarily similar to the mating-type locus in some Dikarya. Intriguingly, the same study observed the presence of two different mating type loci in two *R. irregularis* isolates, while others contained only a single mating type locus. Variant calling combined with single nucleus sequencing and flow cytometry analyzes suggested the presence of two dominant genetically different haploid nuclei, called nucleotypes, in the strains with two mating type loci (Ropars et al., 2016). The other isolates appeared to be homokaryotic, similar to the DAOM197198 isolate (Tisserant et al., 2013; Lin et al., 2014). Interestingly, two of the homokaryotic strains, A1 and B3, contained mating type loci that were found together in the dikaryotic strain A5 (Ropars et al., 2016). This led

the authors to propose a mechanism where two homokaryotic AM fungal strains with compatible mating type loci have the ability to fuse, exchange nuclei and form a stable dikaryotic strain, similar to other heterothallic, bipolar dikarya in the fungal kingdom. The fusion of hyphae (anastomosis) followed by the exchange of nuclei has indeed been reported for genetically closely related AM fungi (Giovannetti et al., 1999, 2015; Croll et al., 2009). However, meiosis or a sexual stage have still not been observed in AM fungi. Many other fungi that were previously thought to propagate only asexually, were later shown to have a cryptic sexual cycle. It can therefore be possible that meiosis is rare in AM fungi or that the right conditions for it to occur (in a natural setting) have not yet been found. Alternatively, the meiosis-related genes may be involved in processes other than meiosis. For example, several meiotic genes have been implicated in processes such as chromatin repair.

If a sexual cycle would not occur, it raises the question how AM fungi have been able to maintain themselves for such a long time. Nuclear fusion and recombination (most notably during meiosis) are generally required to purge the genome of deleterious mutations and to generate genetic variation, creating offspring that would be better adapted to varying environmental conditions. Population genetics approaches suggested the occurrence of (cryptic) recombination in AM fungi (Croll and Sanders, 2009; den Bakker et al., 2010). Additional proof for the occurrence of internuclear recombination was recently claimed by Chen et al. (2018a,b) through single nucleus sequencing of the dikaryotic *R. irregularis* isolate SL1. The recombination in this strain strikingly contrasted with the (almost complete) lack of observed recombination among nuclei of the dikaryons A4 and A5 (Chen et al., 2018a,b). However, it should be noted that the genome coverage of the SL1 strain was very low, and therefore conclusions based on this data set should be handled with care.

Several other hypotheses have been raised as to how genetic variation in AM fungi may contribute to their evolutionary success and ability to adapt to so many environments and plants. One of these hypotheses is based on the level of genetic variation observed within AM fungal individuals (Fig. 20.1D). This is continuing to be a matter of great debate in the AM research community (Kuhn et al., 2001; Sanders and Croll, 2010; Limpens and Geurts, 2014, 2010; Ropars and Corradi, 2015; Young, 2015). Genome sequencing has revealed a relatively low level of genetic variation (0.1–0.4 SNP/Kb) within the presumed “homokaryotic” strains and somewhat higher (varying from ~0.8–2.5 SNP/Kb, depending on strain and method) in the “dikaryotic” strains (Ropars et al., 2016; Wyss et al., 2016; Chen et al., 2018a, b). This variation is significantly lower than the variation reported for true heterokaryotic fungi such as *Rhizoctonia solani*, containing approximately 6 to 15 genetically divergent nuclei within a common cytoplasm (Sneh et al., 1991; Hane et al., 2014), and *Coccidioides immitis*, which have SNP frequencies of 15.9 and 23.7 SNP/Kb, respectively (Neafsey et al., 2010). Nevertheless, reports of phenotypic variation between single spores, derived from single AM individual, have been published (Angelard et al., 2010; 2014; Ehinger et al., 2012; Wyss et al., 2016). Single spore lines derived from two anastomosed *R. irregularis* isolates caused up to five-fold differences in host plant performance when colonizing rice, and showed differences in symbiotic gene expression (Angelard et al., 2010, 2014; Colard et al., 2011). Because spore formation is currently the strongest known genetic bottleneck in AM fungal development, this has led to the suggestion that segregation or drift of (more than two genetically different) nucleotypes may occur as spores are formed. Thereby, different offspring spores may obtain different allele frequencies that could cause the observed differences in phenotypes (Sanders and Croll, 2010). In line with this, several approaches such as SSR markers, AFLP or amplicon sequencing, suggested that shifts in allele-frequencies among individual spores do occur (Wyss et al., 2016; Masclaux et al., 2018). It was even suggested that the host plant may induce a shift in allele frequencies (Wyss et al., 2016). It was claimed that transfer of AM lines to a different host plant species induced a higher genome plasticity, and changes appeared to be non-random (Wyss et al., 2016). This led to the proposal that changes in nucleotype frequency might play an important role in the adaptation to a different (plant) environments as an alternative to sexual reproduction. Frequent exchange of nuclei between AM individuals would ensure the maintenance of genetic variation within the populations. However, the AFLP approach used to monitor the genetic changes in this study makes it difficult to corroborate these findings. A suggestion that the host plant may “select” for preferred alleles or nucleotypes was also put forward after comparing two genome assemblies of the same *R. irregularis* DAOM197198 isolate cultured for over a decade on two different host plants. This revealed slight but significant genetic changes (Tisserant et al., 2013; Lin et al., 2014). Building on this assumption, a model of how selection at the nuclei-level could be involved was proposed (Limpens and Geurts, 2014). In this model different nuclei occupying arbuscules would be rewarded or sanctioned depending on the host plant, for example depending on the effectors being expressed, which would in turn influence the allele frequencies in the mycelium and resulting spores. Of course this nuclei-focussed model could also apply to other selection pressures such as conditions in the soil, potentially leading to segregation in the hyphal network.

In line with this model, it was shown that *R. irregularis* can adjust its transcriptome in a host and stage-dependent manner (Zeng et al., 2018). It was shown that even in a shared hyphal network, the fungus can express different

secreted proteins in the intraradical hyphae and/or arbuscules in a host-dependent manner. It would therefore now be interesting to determine whether this also leads to host-dependent allele expression. Combined with single nucleus sequencing this could reveal whether segregation in the hyphal network indeed occurs. Furthermore, it would be highly informative to determine the level of genetic variation in spores obtained directly from the field, as all the AM fungi sequenced to date came from only two sources in the world and have been in culture with a single host for several decades (Koch et al., 2004). This could reveal whether prolonged culture on the same host leads to a loss of genetic diversity in the fungus.

In true asexually propagating species it has been suggested that mutations (due to replication errors or repeat-induced), horizontal gene transfer and chromatin rearrangements can cause sufficient genetic variation to replace the lack of sex (Seidl and Thomma, 2014). Especially the action of transposable elements (TEs) may promote genome evolution. The excision of transposable elements can create double stranded breaks that need to be repaired by either non-homologous end-joining or homologous recombination. This in turn can lead to inversions, duplications and deletions, and TE insertions can affect neighboring gene expression or knock genes out. The relatively low amount of genetic variation among individual nuclei in AM fungi seems to argue against a prominent role for mutations or genome rearrangements. However, it should be noted that single nucleus sequencing in particular can lead to highly fragmented assemblies (Chen et al., 2018b) by which genome rearrangement could be underestimated.

Bdelloid rotifers are a notable example of asexual organisms that survived evolution through horizontal gene transfer (Gladyshev et al., 2008; Debortoli et al., 2016). By intra- and interspecific allele exchange and gene conversion, these organisms can maintain genetic variation without meiotic recombination. Frequent occurrence of anastomosis between different individuals could facilitate this in AM fungi. However, genomic evidence of these processes has not been found.

Comparing the genomes of different *R. irregularis* isolates showed strikingly high levels of genomic variation. Large variation in gene families numbers, variation in active TE numbers, large numbers of isolate-specific genes and even isolate-specific TEs were observed (Chen et al., 2018a). This indicated that different isolates collected from the same field can vary substantially with respect to their function and potential to adapt to different environments. Furthermore, evidence for inter-isolate exchange was observed, potentially involving anastomosis. After anastomosis, AM fungi may exchange mitochondria in addition to nuclei, however stable inheritance of two mitochondrial genomes has not been found (Providencia et al., 2013). Another theory on the ability of AM fungi to adapt to different environments was raised by the observations that rDNA sequences in AM fungi are highly variable, and possibly encode for different ribosome profiles (Sanders et al., 1995; Pawlowska and Taylor 2004; Maeda et al., 2018). Maeda et al. (2018) found that the genome of *R. irregularis* lack the typical highly tandem organization of the rDNA loci in the genome, as observed in most eukaryotes (Gibbons et al., 2015). Instead, *R. irregularis* was found to only have ten non-repeat rDNAs spread over its genome. Furthermore, SNP frequency in rDNAs was significantly higher than in the rest of the genome, at over 4SNPs/kb. The lack of tandem repeat structures causes AM fungi to not amplify rDNA through unequal sister chromatid recombination, allowing mutations in these sequences to remain. Consequently, variation in rDNA sequences would produce different rRNA profiles per nucleus. Maeda et al. (2018) speculate that AM fungi may rapidly adapt to new environments or hosts by translational regulation through alterations in their ribosome compositions. A similar mechanism has been reported in malaria parasites, a species that also switches hosts during its life cycle (Vembar et al., 2016).

20.8 AM-associated microbes

In nature, AM fungi are surrounded by a plethora of other microbes. Often these microbial communities modulate the mycorrhizal symbiosis and may range from parasitic to beneficial associations, and from endosymbionts to loose hyphal associations.

Several AM fungal species, especially ones belonging to the Gigasporaceae, have been shown to contain endosymbiotic bacteria. Such ancient endosymbiotic bacterial associations with fungi are strikingly prominent in Mucoromycota (Bonfante and Desirò, 2017; Pawlowska et al., 2018). Bacterium-like organisms were originally found by electron microscopy in different AM species, such as *G. calidonium*, *Acaulospora laevis* and *Gi. marginata* (Bianciotto et al., 1996). Subsequent molecular studies in Gigaspora have identified these endosymbiotic bacteria as beta-proteobacteria, closely related to the *Burholderia* genus, called *Candidatus Glomeribacter gigasporae* (Bonfante and Desirò, 2017). *CaGg* is vertically transmitted via the spores, but is not an essential partner as AM fungal individuals with or without these endosymbionts can be found. *CaGg* cannot be cultured outside of the fungus indicating their obligate symbiotic nature, which is also in line with their streamlined genomes ranging from 1.34 to 2.36 Mb. *CaGg* has been shown to

affect the metabolism of its host. AM fungi cured for *CaGg* endosymbionts showed reduced hyphal elongation and branching of germinating spores, which correlates with a reduced amount of lipid droplets and fatty acids in the fungus (Lumini et al., 2007; Salvioli et al., 2010). This is accompanied by a higher expression of genes related to beta-oxidation of fatty acids and the pentose phosphate pathway, and lower respiration rates, indicating a role for *CaGg* in enhancing AM fungal energy metabolism to fuel presymbiotic growth (Vannini et al., 2016). Interestingly, strigolactones activate proliferation of both mitochondria and *CaGg*, suggesting a link between the two. A second endosymbiotic bacterium found in AM fungi is an uncultivable mollicute in the taxon of Mollicutes / Mycoplasma-related endobacteria (Naumann et al., 2010; Toomer et al., 2015). It has been placed in the novel genus *Candidatus Moeniiplasma* and named *C. Moeniiplasma glomeromycotum* (CaMg) (Naito et al., 2017). Both *CaGg* and *CaMg* can be found within the same AM fungal individual. Like *CaGg*, *CaMg* cannot be cultured outside of its host, however its role in the biology of its host is less well characterized. Endosymbiotic bacteria closely related to *CaMg* are also found in other Mucoromycota, where in some cases they have been reported to have a negative impact on their host depending on environmental conditions (Pawlowska et al., 2018; Desirò et al., 2018). The high genetic diversity in the *CaMg* populations within AM fungal individuals could be in line with such facultative parasitic behavior of *CaMg*.

In addition to endosymbiotic bacteria, AM fungi contain bacteria that live on, or close to, the fungal hyphae (Bonfante and Anca 2009; Olsson et al., 2017). This is not surprising as a large amount of carbon fixed by plants ends up in the soil through AM fungi. Several bacteria have been found to have a beneficial effect on the AM symbiosis with plants and they have therefore been termed mycorrhiza-helper bacteria (Frey-Klett et al., 2007). These bacteria belong to diverse bacterial taxa and groups, and the mechanisms by which they stimulate AM symbiosis vary, although in most cases the exact mechanisms are not well known. Such mechanisms can include among others: stimulation of spore germination, promotion of hyphal growth, mineral solubilization, detoxification of soil, inhibition of pathogens and modulation of host root architecture or metabolism (through production or modulation of plant hormones). Given their key role in the plants rhizosphere it can be expected that AM fungi in turn may actively (through the secretion of metabolites and nutrients) or passively (through effects on soil structure and pH) modulate the rhizosphere microbiome (Frey-Klett et al., 2007). For example, as mentioned before, it was shown that AM hyphal exudates can promote the growth of phytase-producing bacteria to increase the access to bound P forms (Zhang et al., 2014a,b, 2016). Fructose was shown to be one of the fungal exudates that could activate phosphatase expression and activity of the phosphate solubilizing bacterium *Rahnella aquatilis* (Zhang et al., 2018). However, due to its difficult accessibility and complex nature, the interplay of AM fungi with other microbes and its environment under natural conditions is still largely understudied.

20.9 Conclusion and future directions

In summary, our insight into fungal aspects of AM symbiosis and their molecular components have significantly increased in recent years, with the availability of the first AM fungal genomes. These now offer a stepping stone to further unravel the molecular workings of this key symbiosis. One of the major areas to be studied is to understand what determines the symbiotic efficiency of different plant-fungus combinations in natural settings. Understanding at a molecular level how (epi-) genetic variation within and among AM fungi, and between different plant species or accessions, contribute to the observed phenotypic variation among AM fungi in the effects on plant performance remains one of the key mysteries in AM biology that remain to be resolved. This will also be of paramount importance to select or breed for optimal plant-fungal combinations for use in sustainable agriculture and to realize the widely recognized potential of AM fungi as sustainable biofertilizers.

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Chapter 21

Engineering bacterial ACC deaminase for improving plant productivity under stressful conditions

Davood Saghafi¹, Behnam Asgari Lajayer¹ and Mansour Ghorbanpour²

¹Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran., ²Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran.

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21.1 Introduction

The world's population is growing and food insecurity is an important issue. The global demand for food is undeniable, which is one of the major problems of developing countries. Hence, population growth and land degradation are threatening for sustainable agriculture (Shahbaz and Ashraf, 2013). The world's population is 7 billion, and it is expected to reach 10 billion in 2070 (Goswami et al., 2016). Increase in global food demand causes the increase of cultivated land to produce more for the growing population. Increasing the cultivated area to grow more crops is partially impossible due to increasing pollution/wastes, urbanization, industrialization, soil erosion and limited access to sustainable water. Therefore, increasing agricultural productivity is the only possible way for the coming years (Vaishnav et al., 2017). To increase the yield of agricultural products, the following interventions are suggested: (1) Better management of agricultural lands; (2) Excessive use of chemical fertilizers; (3) Use of plant growth regulators (PGRs); (4) Use of effective herbicides and pesticides; (5) Mechanized agricultural practices; (6) Cultivating stress-resistant crops; (7) Developing the cultivation of genetic modified (GM) crops; and (8) Developing the plant growth-promoting microorganisms (PGPMs) (Hariadi et al., 2011; Glick, 2014; Paul and Lade, 2014). Some of the above solutions are not efficient and cost-effective in the short term. Since our environment has limited resources, therefore, any long-term effective approach to cultivate food crop must be sustainable and environmentally friendly. Apart from this problem, agricultural productivity is reduced due to biotic and abiotic environmental stresses such as salinity, drought, pH, temperature, heavy metal and organic pollutants, water logging and phytopathogens (Ladeiro, 2012). The role of these stresses in the reduction of growth for the most crop plants is estimated to be more than 50% (Bray et al., 2000). Therefore, it is necessary to be double the crop production for feeding the world population.

Under stress, plants elevate the endogenous levels of ethylene more than its threshold level. In this case, the produced ethylene is named as “stress ethylene” which inhibits plant growth (Singh et al., 2015). Recently, the use of plant growth-promoting microorganisms (PGPBs) as an effective tool for agricultural operations has attracted considerable attention (Goswami et al., 2016). These microorganisms may increase the growth of different plants by various direct mechanisms such as enhancing nitrogen fixation and nutrient availability such as phosphorus, potassium, iron and zinc, as well as production of phytohormones such as auxin (Vaishnav et al., 2017). The indirect procedures comprised of reducing the negative effects of ethylene production through the synthesis of ACC deaminase (Glick, 2014; Choudhary et al., 2015), control of plant pathogenic agents through the production of HCN, chitinase, glucanase (Goswami et al., 2016), mitigate the biotic and abiotic stresses by volatile organic compounds (VOCs) (Tyagia et al., 2018) and induce systemic resistance (ISR) in host plants (Chaudhary and Shukla, 2019). In this context, the ACC-deaminase-containing bacteria through rhizospheric, endophytic and symbiotic interactions with plants can reduce the deleterious effect of environmental stressors (Glick, 2014). The ability of these bacteria to ameliorate the inhibitory effects of ethylene on plant growth has been documented in several studies (Glick 2014; Singh et al., 2015; Saghaei et al., 2018). Therefore, screening and isolating PGPMs containing ACC deaminase and exploiting their ability to stimulate plant growth under stress conditions need further study. In the present review, we focused on: (i) ethylene biosynthesis in plants; (ii) biochemical properties of ACC deaminase and mechanism of its expression; (iii) the role of ACC-deaminase-containing bacteria in improvement plant growth under abiotic and biotic stresses; and (iv) the application of ACC- deaminase in biotechnology.

21.2 Ethylene biosynthesis in plants

In plants, the biosynthesis of ethylene begins with activity of ACC synthase that converts the S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC). In the second step, the ACC oxidase enzyme converts the ACC to ethylene (Fig. 21.1A) (Singh et al., 2015). The hormone ethylene, in a low concentration (10 g L^{-1}), is an important modulator for plant tissues growth such as seed germination, roots, stems, leaves, flowers and fruits, stages of plant development, nodulation of legumes by rhizobia and rooting of cuttings (Glick, 2014; Singh et al., 2015). Also, the environmental factors such as temperature, light, gravity, nutrition, the presence of other plant hormones such as IAA, and biological stresses affect the synthesis of ethylene in plants. In addition, it has been reported that the plants by increasing ethylene levels respond to the metals, organic and inorganic pollutants, temperature extremes, drought and water logging, ultraviolet light, insect and nematode damage, phytopathogens (both fungi and bacteria), and mechanical wounding stresses (Glick, 2014; Singh et al., 2015). When the concentration of ethylene increases to about threshold

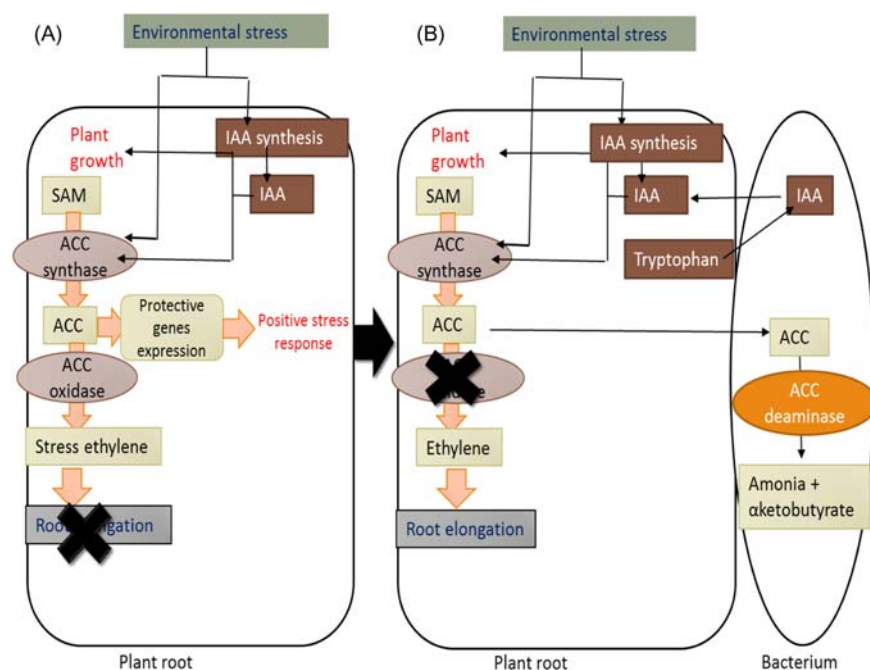


FIGURE 21.1 A schematic model for (A) ethylene biosynthesis pathway within stressed plants, (B) how ACC deaminase containing bacteria improve plant growth under stress conditions.

level (25 g L^{-1}), it negatively affects the plant growth (Singh et al., 2015). In this case, the produced ethylene termed as “stress ethylene”. A model proposed that the synthesis of “stress ethylene” occurs in 2 peaks (Glick et al., 2007). In the first peak, which is very small than the second peak, plants utilize the ACC produced in stressed tissues for activating the expression of genes involved in plant defensive/protective proteins. Subsequently, when the additional ACC synthesized in stressed plants, the second peak of ethylene occurs (generally several days after the initiation of the first peak). In contrast with the first peak, the second one has inhibitory effect on plant growth and it emerges with symptoms such as senescence, chlorosis and leaf abscission (Singh et al., 2015). However, the utilization of ACC deaminase containing bacteria can ameliorate the detrimental effect of ethylene on plant growth by degradation of ACC and lowering the second ethylene peak. It is noteworthy that, in this chapter we only focused on bacterial ACC deaminase for two reasons: (1) it has been suggested that the frequency of bacteria is more than other microorganisms. For example, in 1 g soil exists approximately 9×10^7 bacteria, 4×10^6 actinomycetes, 2×10^5 fungi, 3×10^4 algae, 5×10^3 protozoa and 3×10^1 nematodes (Alexander 1991); (2), although the existence of ACC deaminase has been documented in eukarya, bacteria, and archaea, the majority of ACC deaminase activity is present in different species of bacteria (Nascimento et al., 2014) (Table 21.1). Therefore, in the following sections we discussed about molecular aspects of bacterial ACC deaminase and applications.

21.3 ACC-deaminase

21.3.1 A model of plant growth promotion

Ethylene regulates processes such as root growth and root hairs formation, germination, seed dormancy, fruit ripening, flower senescence and leaf abscission. This hormone also plays several roles in responding to environmental stresses and contributes to crop yields by reducing the growth of stems and roots. Ethylene is produced under various environmental perturbations such as high temperature, flooding, drought, toxic metals, organic pollutants and high salinity (Etesami et al., 2015; Jha and Saraf, 2015). Plants respond to stress through producing ACC, a precursor of ethylene production (Glick et al., 2007). ACC is secreted into the rhizosphere and is again absorbed by the roots and eventually converted to ethylene. The accumulation of ethylene prevents root growth, which consequently limits the water and nutrient uptake. Therefore, any factor that can modify the concentration of ethylene can regulate the growth and development of plants. PGPBs with the ability to use plant ACC as a source of nitrogen and energy in the rhizosphere, convert it into ammonia and α -ketobutyrate. They prevent the accumulation of ethylene and provide a healthy root system to cope with environmental stresses (Siddique et al., 2010). Glick et al. (1998) presented a model to describe the role of ACC deaminase producing bacteria in improving plant growth. When ACC is secreted from plant roots, it is hydrolyzed by the ACC deaminase producing bacteria. Therefore, ACC concentration is reduced outside the root and ACC is more secreted. Consequently, the level of ACC in plants is decreased, resulting in reduced amount of ethylene (Fig. 21.1B). In addition, due to the exudation of photosynthetically metabolites ($\sim 5\text{--}30\%$) such as sugars, organic acids and amino acids, the numbers of bacteria in rhizosphere are 10–1000 times more than in the bulk soil (Glick, 2014). Also, these bacteria from tryptophan in root exudates synthesis IAA which is taken up by plants. Thereafter, the bacterial IAA together with plant synthesized IAA can operate in two ways. It may induce cell elongation in plants or induce the transcription of ACC synthase and subsequently increase the ethylene levels in plants (Fig. 21.1B) (Glick et al., 2007; Stearns et al., 2012). Also, the application of the ACC deaminase producing bacteria can reduce the concentrations of ethylene in plants (Saleem et al., 2018). Therefore, treatment of the plants with ACC- deaminase containing bacteria lead to form roots and shoots in stressed-plants.

21.3.2 Enzymology and biochemical properties

For the first time, the ACC deaminase was purified from *Pseudomonas* sp. strain ACP and followed from *Pseudomonas chloroaphis* 6G5 (Klee et al., 1991) and *Pseudomonas putida* GR12-2 strains (Jacobson et al., 1994). According to results of biochemical studies of ACC- deaminase (Glick, 2014; Singh et al., 2015) can propose that (1) the ACC deaminase is located in the cytoplasm while the ACC is found in plant tissues; (2) the K_m value of the enzyme ranged from 1.5 to 15 Mm, indicating that the affinity of ACC deaminase for ACC is very low. ACC deaminase is an inducible enzyme and the minimum level of ACC for induction was reported to be 100 nM in *Pseudomonas* sp. strain ACP and *P. putida* GR12-2 strains. For measuring the ACC deaminase activity, the bacterial strains are grown on minimal medium containing ACC. Also, a basal level of enzyme activity is measured by growing strains on minimal medium containing ammonium sulfate. In addition to ACC, the activity of ACC deaminase enzyme can induce with other amino

TABLE 21.1 List of bacteria containing ACC deaminase activity.

Strain	ACC deaminase activity (nmol α -K mg ⁻¹ h ⁻¹)	References
<i>Achromobacter xylosoxidans</i> A551	400	Belimov et al., 2005
<i>Acidovorax facilis</i> 4p-6	3080	Belimov et al., 2001, 2005
<i>A. xylosoxidans</i> AF302097	151	Belimov et al., 2001
<i>A. xylosoxidans</i> Bm1	90	Belimov et al., 2005
<i>Alcaligenes</i> sp. AF288728	1172	Belimov et al., 2001
<i>A. xylosoxidans</i> AF302096	555	Belimov et al., 2001
<i>A. xylosoxidans</i> AF288734	305	Dell'Amico et al., 2008
<i>Bacillus pumilus</i> AF288735	760	Belimov et al., 2001
<i>B. pumilus</i> SB1-ACC3	1460	Bal et al., 2013
<i>B. licheniformis</i> B2r	860	Chookietwattana and Maneewan, 2012
<i>Bacillus</i> sp. MR4	15,920	Grobelak et al., 2018
<i>Bacillus cereus</i> LB1	2400	Hemida and Reyad, 2018
<i>Bacillus aerius</i> SB1	1800	Hemida and Reyad, 2018
<i>B. subtilis</i> LK14 and LK15	454,310	Khan et al., 2000
<i>Burkholderia caryophylli</i>	598	Shaharoona et al., 2007b
<i>Enterobacter cloacae</i>	295	Nadeem et al., 2010
<i>Enterobacter</i> sp. CS1	170	Huang et al., 2013
<i>E. aerogenes</i>	341	Nadeem et al., 2007
<i>Flavobacterium ferrugineum</i>	405	Nadeem et al., 2007
<i>Methylobacterium</i> sp.	94	Madhaiyan et al., 2006
<i>Mycobacterium</i> sp.	1.14	Dell'Amico et al., 2008
<i>Pseudomonas aeruginosa</i>	153	Zahir et al., 2009
<i>P. aeruginosa</i> SLC-2	2520	Liu et al., 2015
<i>Pseudomonas</i> sp. ST3	900	Trung et al., 2016
<i>Pseudomonas</i> sp. R3	23,490	Grobelak et al., 2018
<i>P. bathycetes</i>	501	Nadeem et al., 2007
<i>P. tolaasii</i>	1.16	Dell'Amico et al., 2008
<i>P. brassicacearum</i> AY007428	972	Belimov et al., 2001
<i>P. chlororaphis</i>	456	Nadeem et al., 2007
<i>P. fluorescens</i>	421	Nadeem et al., 2007
<i>P. fluorescens</i> TDK1	349	Zahir et al., 2009
<i>P. fluorescens</i> biotype F	342	Zahir et al., 2008
<i>P. syringae</i>	440	Nadeem et al., 2007
<i>Rhizobium hedysari</i> ATCC43676	20	Ma et al., 2003b
<i>R. leguminosarum</i> 128C53K	5	Belimov et al., 2001, 2005
<i>R. leguminosarum</i> 99A1	430	Ma et al., 2003a
<i>Mesorhizobium ciceri</i> strain LMS-1 (pRKACC)	2035	Nascimento et al., 2012
<i>Sinorhizobium meliloti</i> KYA71 and KYA40	326,136	Khosravi et al., 2014
<i>Rhodococcus</i> sp. AF288731	833	Belimov et al., 2001
<i>Rhodococcus</i> sp. strainFp2	7320	Belimov et al., 2001, 2005
<i>Rhodococcus</i> sp. strain4N-4	12,970	Belimov et al., 2001, 2005
<i>Serratia ficaria</i>	326	Nadeem et al., 2010
<i>S. marcescens</i> BC-3	38,520	Liu et al., 2015
<i>S. proteamaculans</i>	276	Zahir et al., 2009
<i>S. quinivirans</i> SUD165	12	Belimov et al., 2001, 2005
<i>Variovorax paradoxus</i> 3P-3	3700	Belimov et al., 2001, 2005
<i>V. paradoxus</i> 5C-2	4322	Belimov et al., 2001, 2005
<i>V. paradoxus</i> 2C-1	3588	Belimov et al., 2001, 2005
<i>V. paradoxus</i> sp.	1805	Belimov et al., 2005

acids such as L-alanine, L-serine, dl-alanine, dl-valine, L- homoserine and L- α aminobutyric acid; (3) the optimum activity of enzyme is observed in 25–30 °C and 8.0–8.5 for temperature and pH, respectively; and (4) the form of enzyme is trimeric and utilize pyridoxal 5-phosphate as co-factor for activity. In this case, the ratio of pyridoxal 5-phosphate

bounded to ACC deaminase is 3:1. For *P. sp.* strain ACP the native molecular mass of enzyme has been reported about 110–112 kDa, and for *P. putida* GR12-2 ACC deaminase stated to be 105 kDa. The form of enzyme is trimeric and has an approximate subunit mass of 36.5 and 35 kDa for *P. sp.* strain ACP and *P. putida* GR12-2, respectively (Singh et al., 2015).

21.3.3 Mechanism of ACC -deaminase expression

The *acdS* gene isolated from proteobacteria (α , β and γ), Firmicutes, Actinobacteria and members of Deinococcus-Thermus phylum (*Meiothermus*) (Nascimento et al., 2014). However, the existence of *acdS* gene has not been confirmed in 271 completely sequenced strains belonging to Firmicutes phylum. The possible reason for this can be horizon gene transfer among bacteria (Nascimento et al., 2014). However, in case *Meiothermus ruber* (a thermophile bacterium with optimum growth at 60 °C), the existence of the *acdS* gene has been explained through the vertical transmission of this gene. Also, the existence of *acdS* gene in *Agreia* sp. PHSC20C1 (a psychrophile marine actinobacterium) has been confirmed with occurrence of vertical transmission gene. However, the *acdS* gene of *Azorhizobium* and *Bradyrhizobium* strains is located away from chromosomal symbiotic (Nascimento et al., 2014). In β Proteobacteria, such as *Burkholderia* and *Cupriavidus* genus and *Agrobacterium* and *Rhizobium* strains the *acdS* gene is located on a second chromosome. Similarly, it is documented the *acdS* gene in *Azorhizobium*, *Bradyrhizobium* and *Gluconacetobacter xylinus* NBRC 3288 is located on the primary chromosome. However, in other bacteria such as Rhizobiaceae (*Rhizobium*, *Sinorhizobium*, and *Agrobacterium*), Phyllobacteriaceae (*Phyllobacterium* and *Mesorhizobium*) and *Azospirillum* strains the *acdS* genes are located on plasmids or second chromosomes (Nascimento et al., 2014).

A study by Prigent-Combaret et al. (2008) reported that an LRP homolog (*acdR*) located near the *acdS* gene 45 of 48 studied Proteobacteria. However, from 261 tested Proteobacteria, in 166 strains have been found the existence of *acdR* near the *acdS* gene (Nascimento et al., 2014). Moreover, in many studied Proteobacteria the transcription of *acdR* and *acdS* genes are found in the opposite direction (Prigent-Combaret et al., 2008; Nascimento et al., 2014). This information indicates that the evolution of *acdR* and *acdS* genes perform in similar way (Nascimento et al., 2014). However, the *acdR* gene was not found in all microorganisms. In a study, from 17 positive strains (6 *Mesorhizobium* strains, 2 *Rhizobium* strains, *Fulvimarina pelagi*, 3 root nodule *Burkholderia* strains, *Halomonas titanicae* BH1 and 4 *Pseudomonas* strains) for *acdS*, was not found the *acdR* gene (Nascimento et al., 2014). Also, in *Burkholderia xenovorans* LB400 was observed the *acdR* gene away from the *acdS* gene (Onofre-Lemus et al., 2009). However, it has been reported that the *acdR* gene was not observed in *Rhizobium leguminosarum* bv. *viciae* 3841 and *Mesorhizobium loti* MAFF303099 (Uchiumi et al., 2004). From the aforesaid information, we can conclude the possible reason for the absence of *acdR* genes in *acdS* positive strains is the occurrence of genome rearrangements or gene insertions (Nascimento et al., 2014). For example, there are two copies of the *acdS* gene in *Burkholderia* sp. CCGE1002, *B. phyatum* STM815 and *B. phenoliruptrix* BR3459a strains. In contrast, Checcucci et al. (2017) reported that in *Sinorhizobium meliloti* the expression of *acdS* gene is repressed with the *acdR*-like gene. Therefore, regarding *acdR* negative genes in most proteobacteria the expression of ACC deaminase indicates the presence of other elements such as *CRP* and *FNR* binding sites in the upstream region of the *acdS* gene (Grichko and Glick, 2000; Prigent-Combaret et al., 2008). In *Mesorhizobium loti* MAFF303099, the expression of ACC deaminase regulates the NifA protein (Singh et al., 2015). In addition, it has been found a NifA binding site in the upstream region of the *acdS* gene in other *Mesorhizobium* strains (Nascimento et al., 2012). Also, the expression of ACC deaminase in Actinobacteria and *Meiothermus* regulate *acd-AR* and *acd-MR* genes, respectively (Nascimento et al., 2014).

In the case of *acdS* and *acdR* genes, the most studies have been focused on Proteobacteria. However, precise mechanism of *acdS* gene regulation in different strains has not been well identified yet (Singh et al., 2015). However, Li et al. (2000) proposed a model for the regulation of *acdS* gene in *P. putida* UW4 bacterium. This model consist of *acdR* gene suggested, Lrp box for binding Lrp protein, AcdB box for binding AcdB protein, FNR box (for fumarate and nitrate reductase protein binding), CRP box for cAMP receptor protein binding and promoter regions for boxes binding. Under stress, when the ACC is present, active form of LRP along with ACC and AcdB by building triparental complex and binding to promoter region of *AcidS* regulate its expression. However, the activity of ACC deaminase is regulated negatively via leucine (by product of α -ketobutyrate), causing increased leucine concentration and repression of *acdS* gene through the formation of inactive LRP. However, the regulatory mechanism of *acdS* varies among the different strains. A model of *acdS* gene expression has been proposed in Rhizobia bacteria (especially in *M. loti*) (Nukui et al., 2006). In this model, N_2 fixing units (*nifA*₁ and *nifA*₂) and σ^{54} (RNA polymerase sigma recognition site) are located on upstream of *acdS* and *nifH* genes. According to the model, *nifA*₂ encodes protein NifA₂ which interacts with σ^{54} and then favors

for *acdS* gene expression. In a study, [Khosravi et al. \(2014\)](#) evaluated the ACC deaminase activity of 168 isolates belonging to *Sinorhizobium meliloti*. According to growth rate on ACC-containing medium and enzyme activity, these isolates were classified into four groups. Two isolates namely KYA40, and KYA71 (high positive for ACC deaminase activity on plate medium) were selected for molecular characterization. They found that the ACC deaminase genes (*acdS*) of KYA40, and KYA71 strains located on mega plasmids and chromosomal DNA, respectively. Also, the comparison of amino acid sequences of KYA40 and KYA71 with other sequences in the database showed that the amino acids 37–58 in almost all strains were similar and any changes in this region of *acdS* gene may alter the activity of ACC-deaminase. Similarly, [Todorovic and Glick \(2008\)](#) reported that mutations in ACC deaminase amino acids (E295S/L322T) in *Pseudomonas* sp. UW4 cause to the loss of ACC deaminase ability.

21.3.4 Nodulation

The efficiency of rhizobia strains containing ACC deaminase in nodules formation is more than the negative ACC deaminase strains (up to 40%) ([Ma et al., 2003a, 2004](#)). In other study, [Ma et al. \(2004\)](#) reported that *S. meliloti* containing ACC deaminase increased the nodulation *Medicago sativa* plants. However, compared with free-living plant growth-promoting bacteria, the amount of ACC deaminase produced by rhizobia strains is up to 2–10%. Therefore, free-living bacteria by producing a high level of ACC deaminase in host plants (mainly roots) and subsequently by decreasing ethylene levels in the plant tissues can protect them from adverse conditions ([Glick 2014](#)). In contrast, the rhizobia strains specifically bind to the roots of host plants, and locally reduce the ethylene levels only within nodules. Further studies showed that the transcription of *acdS* genes together with the transcription of *nif* and nitrogen fixation genes regulated by transcription of *nifA* promoter take place in various strains of *Mesorhizobium loti* ([Uchiumi et al., 2004; Nukui et al., 2006; Nascimento et al., 2012](#)). However, the expression of ACC deaminase in *M. loti* and *S. meliloti* does not stimulate nodulation. Therefore, ACC deaminase is responsible for root colonization and endophyte behavior in *S. meliloti* ([Checcucci et al., 2017](#)), while it reduce the rate of nodule senescence in *M. loti* ([Glick 2014](#)). Due to the utilization of energy in nitrogen fixation process that cause to synthesis of stress ethylene, this mode of action of ACC deaminase has been observed in *M. loti*.

Efficiency of biofertilizers based on ACC deaminase producing bacteria has been well proven to improve plants growth under environmental stress conditions ([Ahmad et al., 2013a; Nadeem et al., 2014](#)). In a study, the effectiveness of ACC deaminase containing bacteria (*Pseudomonas* sp. and *Bacillus* sp.) in combination with *Mesorhizobium cicri* has been tested for improving the yield of field grown chickpea plants under water stress. The results showed that the inoculation of multi-strain PGPR significantly increased the nodulation (such as number, fresh and dry weight of nodules) of chickpea plants ([Ahmad et al., 2017](#)). It is noticeable that efficiently root colonization by rhizobia strains inoculation could be the main reason for the increment of nodulation ([Verma et al., 2012](#)). Also, ACC deaminase containing bacteria by increasing root growth and its proliferation, and subsequently by increasing root surface area under stress conditions ([Ahmad et al., 2011](#)) provide more sites for attachment of rhizobia strains to make nodules on chickpea roots. Similarly, [Ahmad et al. \(2013b\)](#) reported that the usage of *rhizobium* in combination with PGPR as biofertilizer under saline field conditions, lead to increase the nodulation of mung bean plants.

21.3.5 Environmental perturbations

Commonly, ACC deaminase-producing microorganisms live in soil. In addition, ACC deaminase genes are found in microbial isolated from different sources such as sea, hot springs and industrial sludge. It has been reported that the gram-negative bacteria ([Belimov et al., 2001; Tak et al., 2013](#)), gram-positive bacteria ([Belimov et al., 2001; Timmusk et al., 2011](#)), rhizobia ([Ma et al., 2003b; Uchiumi et al., 2004](#)), and endophytes ([Rashid et al., 2012](#)) are able to express ACC deaminase. The presence of *acdS* gene is important in plant-microbe interactions ([Gamalero and Glick 2012; Singh et al., 2015](#)). For example, *P. putida* GR12-2 by the ACC deaminase activity obtains competitive properties against rhizosphere microorganisms ([Jacobson et al., 1994](#)). The existence of *acdS* gene in *S. meliloti* increased the competitiveness properties compare with wild-type strain ([Ma et al., 2004](#)). Similarly, [Checcucci et al. \(2017\)](#) reported that the *acdS* gene is important for root colonization and involved in the endophytic behavior in *S. meliloti*. For the first time, [Ma et al. \(2003b\)](#) tested the ACC deaminase activity in 13 rhizobia strains. They showed that 5 of 13 strains possess ACC deaminase activity while 7 of 13 strains possess *acdS* gene. Furthermore, the existence of *acdS* genes in these strains (negative for ACC deaminase activity) indicates that the vertical transfer of *acdS* genes has occurred. [Duan et al. \(2009\)](#) screened the ACC deaminase activity of 233 rhizobia strains isolated from 30 different soils. In their study,

27 of 233 strains indicate ACC deaminase activity. Etesami et al. (2014) reported that out of 80 endophytic bacteria isolated from rice roots, 28 strains were positive for ACC deaminase.

It is assumed that the number of ACC positive bacteria increased under stress conditions, compare to normal conditions. In a study, Timmusk et al. (2011) tested the existence of ACC deaminase in bacterial strains isolated from the wild barley (*Hordeum spontaneum*) rhizosphere in two directions (south-and north-facing slopes). According to the results, 50% of the bacteria isolated from south-facing slope were positive ACC deaminase activity while 4% of the bacteria isolated from north-facing slope were positive. Also, Nascimento et al. (2014) reported the most of ACC deaminase containing bacteria were isolated from extreme environments. In addition, it has been suggested that the abundance of ACC positive bacteria depend on plant genotype. To date, studies about these bacteria were performed based on their isolation and inoculation on plants growth under the greenhouse or field conditions (Saleem et al., 2007; Forni et al., 2017), however, the number of studies conducted on the natural abundance and diversity of indigenous ACC positive populations in rhizospheres are rare (Timmusk et al., 2011). Recently, two field studies were conducted to evaluation the abundance, composition, and ACC deaminase activity of ACC positive bacteria in different winter wheat (*Triticum aestivum* L.) genotypes (Baca, Hatcher, Ripper and Ronl) under different irrigation regimes (Stromberger et al., 2017). The results showed that the relative abundance of ACC positive bacteria in wheat rhizospheres ranging from 1.69×10^7 to 3.28×10^9 CFUs g⁻¹ and increased when the soil water content decreased. Relative abundance was also affected by genotype, with the greatest percentage of ACC positive bacteria in the rhizosphere of 'RonL' grown with limited irrigation. Mostly, species composition included *Pseudomonas* (35–54%), *Sphingobacterium* (3–13%), *Chryseobacterium* (5–10%), *Buttiauxella* (4–11%), *Stenotrophomonas* (~4%), and *Acinetobacter* (0–5%). Also the distributions of these bacteria varied between Ripper and Ronl wheat genotypes. Therefore, according to results the RonL (drought tolerance genotype) rhizosphere had the greatest abundance of *acdS* gene and ACC deaminase activity, compared with other genotypes (Stromberger et al., 2017). Similarly, Bouffaud et al. (2018) evaluated the effects of maize and other *Poaceae* species on diversity of ACC deaminase producing bacteria (based on size of *acdS* gene and number of *acdS* transcripts). According to results, the size of *acdS* gene amounted to $0.5\text{--}6.2 \times 10^6$ *acdS* gene copies $0.5\text{--}2 \times 10^7$ per g of rhizosphere soil in the cropped soil and in the meadow soil at 21 days, respectively. Also, the number of *acdS* transcripts reached $0.06\text{--}2.9 \times 10^3$ to $0.08\text{--}2.1 \times 10^3$ *acdS* cDNA copies per g of rhizosphere soil in the cropped soil and in the meadow soil at 21 days, respectively. Compared to the bulk soil, *acdS* group size and number of *acdS* transcripts were higher in the presence of plant. Results indicate the ACC deaminase positive bacteria adapt to survive under stressed conditions by interaction with roots of plant host.

21.4 Amelioration of environmental stresses

The efficiency of ACC deaminase producing bacteria to enhance the stressed-plants growth was proven (Etesami et al., 2014; Han et al., 2015; Saleem et al., 2018; Saghafi et al., 2018, 2019a). For example, rhizobia containing ACC deaminase genes can increase the nodule formation and subsequently N₂ fixation in many legume plants, under stress conditions (Ma et al., 2003a, 2004). In other case, the ability of ACC deaminase producing bacteria in root colonization have been documented (Checcucci et al., 2017). However, the inoculation of stressed-plants with ACC deaminase producing bacteria results in the increase of plant tolerance to the both biotic and abiotic stresses (Table 21.2). The following section discusses the role of ACC deaminase producing bacteria in amelioration of different stresses against plants.

21.4.1 Flooding

The plant roots synthesize a high levels of ACC under flooding (hypoxic or oxygen limited) stress, and due to lack of O₂ in roots, the ACC is transported to shoots and converted to ethylene by ACC oxidase enzyme (Gamalero and Glick 2015). The ethylene biosynthesis by stressed-plants cause symptoms such as epinasty (wilting), leaf chlorosis, necrosis and reduced biomass yield (Li et al., 2013). It has been reported that ACC deaminase containing bacteria are able to protect plants from negatively effects of ethylene produced under adverse conditions (Grichko and Glick, 2001a; Li et al., 2013). In a study, Onofre-Lemus et al. (2009) inoculated the tomato plants with ACC deaminase producing *Burkholderia unamae* bacterium (including wild-type strain namely *Burkholderia unamae*, and *acdS*: 'ΩKm mutant) under flooding stress. The results showed that the inoculation of tomato plants with wild-type strain significantly improved the shoot and root dry weight and chlorophyll content compared to other treatment. In another study, the role of ACC deaminase of *Pseudomonas fluorescens* strain REN1 and its ability to reduce ethylene levels produced during flooding stress, colonize and promote the elongation of roots in rice seedlings was investigated under gnotobiotic conditions (Etesami et al., 2014). They found that the ACC deaminase containing *P. fluorescens* REN1

TABLE 21.2 Examples for the amelioration environmental stresses by bacteria containing ACC deaminase activity.

Bacteria	Host plant	Stress	References
<i>Alcaligenes</i> sp., <i>Bacillus pumilus</i>	<i>Brassica napus</i>	Heavy metals	Belimov et al., 2001
<i>Pseudomonas stutzeri</i> A1501	<i>Oryza sativa</i>	Heavy metals	Han et al., 2015
Combination of <i>Pseudomonas</i> sp., <i>B. cereus</i> and <i>Bacillus</i> sp.	<i>Festuca rubra</i> and <i>Brassica napus</i>	Heavy metals	Grobelak et al., 2018
Combination of <i>Bacillus</i> sp., <i>B. thuringiensis</i> . and <i>Bacillus</i> sp.	<i>Festuca rubra</i> and <i>Brassica napus</i>	Heavy metals	Grobelak et al., 2018
<i>Burkholderia</i> sp.J62	<i>Lycopersicon esculentum</i>	Lead	Jang et al., 2008
<i>Enterobacter. cloacae</i> CAL2	<i>Brassica napus</i>	Arsenate	Nie et al., 2002
<i>P.putida</i> UW4, <i>P.putida</i> HS-2 <i>P</i>	<i>Brassica napus</i>	Nickel	Farwell et al., 2007
<i>Pseudomonasputida</i> UW4	<i>Lycopersicon esculentum</i>	Flooding	Grichko and Glick, 2001a
<i>Pseudomonas fluorescens</i> strain REN1	<i>Oryza sativa</i>	Flooding	Etesami et al., 2014
<i>Bacillus cereus</i> LB1 and <i>Bacillus aerius</i> SB1	<i>Carthamus tinctorius</i>	Salt	Hemida and Reyad, 2018
<i>Enterobacter</i> sp. E5 and <i>Kosakonia</i> sp. S1	<i>Oryza sativa</i>	Salt	Liu et al., 2017
<i>Variovorax paradoxus</i> 5C-2	<i>Pisum sativum</i>	Salt	Wang et al., 2016
<i>Pseudomonas</i> sp. ST3	<i>Vigna unguiculata</i>	Salt	Trung et al., 2016
<i>Bacillus</i> sp., <i>Zhihengliuella halotolerans</i> and <i>Staphylococcus succinus</i>	<i>Triticum aestivum</i>	Salt	Orhan, 2016
<i>Pseudomonas stutzeri</i> A1501	<i>Oryza sativa</i>	Salt	Han et al., 2015
<i>P.fluorescens</i> YsS6	<i>Lycopersicon esculentum</i>	Salt	Ali et al., 2014
<i>P.putida</i> UW4	<i>Lycopersicon esculentum</i>	Salt	Yan et al., 2013
<i>Pseudomonas fluorescens</i>	<i>Arachis hypogea</i>	Salt	Saravanakumar and Samiyappan, 2007
<i>Achromobacter piechaudii</i> ARV8	<i>Lycopersicon esculentum</i>	Salt	Mayak et al., 2004
<i>P.fluorensce</i> ns CHA0	<i>Solanum tuberosum</i>	Plant pathogen	Wang et al., 2000
<i>P.fluorensce</i> ns CHA96	<i>Cucumis sativus</i>	Plant pathogen	Wang et al., 2000
<i>P.putida</i> UW4	<i>Pinus sabiniana</i>	Plant pathogen	Nascimento et al., 2013
<i>Enterobacter</i> HS9 and <i>Bacillus</i> G9	<i>Mucuna pruriens</i>	Drought	Saleem et al., 2018
<i>.fluorescens</i> ACC-5 <i>P</i>	<i>Pisum sativum</i>	Drought	Zahir et al., 2008
<i>Pseudomonas</i> sp.	<i>Pisum sativum</i>	Drought	Arshad et al., 2008
<i>Sinorhizobium</i> sp. Pb002	<i>Brassica juncea</i>	Lead	Di Gregorio et al., 2006
<i>Variovorax paradoxus</i>	<i>Brassica juncea</i>	Cadmium	Belimov et al., 2005
<i>Pseudomonas aeruginosa</i> SLC-2 and <i>Serratia marcescens</i> BC-3	<i>Avena sativa</i>	Petroleum pollutant	Liu et al., 2015

increased in vitro root elongation and endophytically colonized the root of rice seedlings as compared to control under constant flooding conditions.

21.4.2 Drought

Under water stress, the physiological and morphological changes such as photosynthesis (A), stomatal conductance (gs), carbon metabolism, water relations and hormone production occur in plants for regulating below- and above-ground parts growth (Ghassemi et al., 2018; Khoshmanzar et al., 2019). Also, plants release chemicals signals such as abscisic acid, ACC, ethylene under drought stress. However, one of the most common responses is increase in ethylene production (Saleem et al., 2018). Previous studies have documented that PGPR containing ACC deaminase activity reduce level of the stress hormone ethylene within plant tissues such as (pea [*Pisum sativum*], maize, wheat, mung bean [*Vigna radiata*], and *Trigonella foenumgraecum*) may improve plants growth under drought stress conditions

(Arshad et al., 2008; Belimov et al., 2009; Shakir et al., 2012; Barnawal et al., 2013; Sarma and Saikia 2014; Zafarul Hye et al., 2014). To mitigate drought stress, an experiment was conducted by using ACC deaminase containing plant growth promoting rhizobacteria (PGPR) for improving chickpea crop production (Shan, 2017). In this study, 47 bacterial strains belonging to genus *Bacillus* (25 strains) and *Pseudomonas* (22 strains) were isolated from different rhizospheric soils of chickpea in Punjab (India). Ten of the isolates were able to utilize ACC as a sole source of nitrogen, maximum growth (in terms of optical density OD600) being recorded with *Bacillus* isolate 23-B (0.463) followed by *Pseudomonas* 6-P (0.317). Two PGPR's 23-B and 6-P alone and in combination with recommended (for Punjab state) *Mesorhizobium ciceris*, were evaluated for water stress mitigation and plant growth promotion under axenic conditions on *Cicer arietinum* varieties (Kabuli L-552 and Desi GPF-2). Both the rhizobacteria (especially *Bacillus* isolate 23-B) significantly improved germination, root and shoot length and fresh weight of chickpea seedlings under osmotic potential of 0.4 MPa, compared to noninoculated control. Also, co-inoculation of 23-B with *Mesorhizobium* enhanced all growth parameters under water deficit stress. Similarly, the velvet bean (*Mucuna pruriens* L.) plants were inoculated by ACC deaminase positive rhizobacteria (*Enterobacter* spp. (HS9) and *Bacillus* spp. (G9)) under drought stress (Saleem et al., 2018). According to the results, significant increase of root (25–27%) and shoot (18–28%) length, and shoot and root dry weight (70–78%) and the number of leaves were observed in inoculated plants compared to controls. Also, the inoculation by PGPR showed that the concentration of ACC and ethylene negatively affected in leaves and roots. However, the ACC content in leaves of inoculated plants was reduced by 41%, while the ACC content in roots decreased to 46%. Also, a similar trend was observed for ethylene concentration in leaves and roots of inoculated plants, where maximum decrease of 65% and 72% were found, respectively.

21.4.3 Salinity

A soil is considered saline when the electrical conductivity (saturated extract EC) is more than 4 dS m^{-1} ($\sim 40 \text{ mM NaCl}$). This level of salinity is critical to start reducing the productivity of many crops (Jamil et al., 2011). Various types of salts, such as NaCl , Na_2SO_4 , MgSO_4 , MgCl_2 , are present in saline soils. However, NaCl is more damaging for plants than others (Jamil et al., 2011). Salinity is the accumulation of water-soluble salts, which included K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , CO_3^{2-} , HCO_3^- , and Na^+ ions. Depending on the soil, the concentration of soluble salts is different (Vaishnav et al., 2017). Mineralization is the main source of soils salinity. Human activities such as irrigation of plants with saline water or evaporation of groundwater (due to inadequate precipitation, ions are not leached from the soil profile) are some other sources of soils salinity (Vaishnav et al., 2017). It is estimated that 20% of the total cultivated land and 33% of the irrigated agricultural lands around the world are affected by salinity. If this trend continues, 50% of the cultivated land will be saline by 2050 (Vaishnav et al., 2017).

Salinity reduces plant growth and development by changing the biochemical and physiological processes such as leaf growth, root growth, water absorption, stomatal conductance and photosynthesis (Patil, 2013). Among above mentioned indices, photosynthesis is closely related to plant growth and yield. This index includes the parts of CO_2 that is assimilated, photosynthetic electron and proton transport rate (Tang et al., 2014). The presence of salts in the root zone creates osmotic (water deficiency) and ion stresses for plants (Paul and Lade, 2014). Consequently, some processes such as photosynthesis and cell division are affected under stressful condition (Yan et al., 2013). Salt stress by reducing stomatal and mesophyll conductance and CO_2 availability, decreases the photosynthetic activity. Moreover, salt stress indirectly can affect photosynthesis by reducing chlorophyll and total carotenoids content. The photosynthesis rate is measured by weighing gas exchange and chlorophyll a fluorescence (Yan et al., 2013; Tang et al., 2014). Some plants change the photosynthetic pathway to cope with salinity stress. For example, the *M. crystallinum* plants by transforming from C_3 pathway to CAM adapts to water deficiency in saline environment. *Atriplex lentiformis* by shifting from C_3 to C_4 may cope with the salt stress damage (Tang et al., 2014). Gu et al. (2016) stated that the ratio of root to shoot biomass is an indicator tolerance of plants to salinity stress. The greater value of this ratio, the higher resistance of plants to stress. Also, high concentrations of sodium and chloride lead to ion-specific toxicity and ion imbalance (Gu et al., 2016). Under these conditions, the absorption of K^+ , NO_3^- , PO_4^{2-} , Ca^{2+} , Mg^{2+} and consequently, the ratios of K^+/Na^+ , $\text{Ca}^{2+}/\text{Na}^+$, $\text{Mg}^{2+}/\text{Na}^+$, $\text{PO}_4^{2-}/\text{Cl}^-$ and $\text{NO}_3^-/\text{Cl}^-$ decreases (Tester and Davenport 2003; Gu et al., 2016). In addition, the production of reactive oxygen species (ROS) (Yan et al., 2013; Tang et al., 2014) and the concentration of ethylene increase under salinity stress (Patil, 2013). However, it is reasonable to employ the ACC deaminase-containing bacteria for lowering ethylene concentration in plants under such conditions. For the first time, Mayak et al. (2004) successfully tested the ability of *Achromobacter piechaudii* ARV8A for improving plant growth under salt stress. Then, the application of ACC deaminase-containing bacteria was received more attention by other researchers (Ali et al., 2014; Barnawal et al., 2014; Chang et al., 2014; Saghaei et al., 2018, 2019b).

Han et al. (2015) inoculated the rice seedling with ACC deaminase containing *Pseudomonas stutzeri* A1501 (Wild-type) and A1815 (negative for ACC deaminase activity) under 0.12 M and 2 M NaCl. According to their results, inoculation with the *P. stutzeri* A1501 strain significantly improved plant growth parameters such as the plant height, root length, root fresh weight, and root dry weight, compared to the control plants. On the contrary, these abilities were not observed in the mutant strain (*Pseudomonas stutzeri* A1815), and located in same class with control plants without any significant differences ($P < 0.05$). These finding implicate the role of the *acdS* gene in the *P. stutzeri* A1501 for plant growth-promoting trait. Furthermore, under 120 mM NaCl, the A1815-inoculated rice seedling showed lower plant height (by 20%), root length (by 30%), root fresh weight (by 22%), and root dry weight (by 28%) compared with plants inoculated with *P. stutzeri* A1501. Also, these changes in the growth parameters were more significant under 2 M NaCl. In a study, the cowpea seedlings were inoculated with salt tolerant ACC deaminase-producing bacterium namely *Pseudomonas* sp. ST3. Cowpea plants inoculated with ST3 strain revealed a significant increase in shoot length and shoot fresh weight over uninoculated control at the salinity level of 1.5% NaCl (Trung et al., 2016). Similarly, inoculated pea plants by *Variovorax paradoxus* 5C-2 bacterium (ACC deaminase-producing) increased total biomass, shoot K^+/Na^+ ratio, photosynthetic efficiency (Fv/Fm) and maximum electron transport rate (ETR) under salt stress (Wang et al., 2016). Recently, Hemida and Reyad (2018) inoculated the *Carthamus tinctorius* plants with two PGPRs (ACC deaminase-producing) namely *Bacillus cereus* and *Bacillus aerius* under salinity stress. The results showed that in inoculated plants total biomass (dry and fresh weight) and the activity of catalase, ascorbate peroxidase and glutathione reductase enzymes, also glutathione and ascorbic acid was significantly higher than those of control plants. More recently, Saghafi et al. (2019a) also reported that ACC deaminase-producing halotolerant bacteria can improve the growth of *Helianthus annuus*, *Triticum aestivum*, *Brassica campestris*, *Bassia indica*, *Catharanthus Roseus* and *Brassica napus* plants under salt stress. Similarly, Saghafi et al. (2019b) were inoculated canola (*Brassica napus* L.) seedling with four ACC deaminase-producing halotolerant *Rhizobium* strains, R29 (*Sinorhizobium mellilote*), R103 (*Sinorhizobium mellilote*), R281 (*Rhizobium leguminosarum* bv. *phaseoli*) and R307 (*Rhizobium leguminosarum* bv. *phaseoli*) under salt stress (50 mM). They reported that inoculation of *Rhizobium* strains significantly increased the shoot/root elongation, shoot/root dry weight, stem diameter, leaf numbers, RWC, salt tolerance index (ratio of biomass of salt stressed to non-stressed plant) and nutrient uptakes (N, P, K) compared to positive control (50 mM salt). The same authors indicated that use of ACC- deaminase producing halotolerant *Rhizobium* bacteria mitigates the negative salt stress effects.

21.4.4 Flower wilting

Almost, in plants the senescence of flowers occurs by excessive ethylene production. Therefore, application of chemical ethylene inhibitors such as silver thiosulfate is useful for increasing shelf life of cut flowers. However, these chemical substances are harmful for environment (Gamalero and Glick 2015). The treatment of cut flowers with ACC deaminase-producing rizhobacteria could be an environmentally friendly alternative approach compared to silver thiosulfate (Ali et al., 2012). In a study, the treatment carnations cut flowers by ACC deaminase-producing rizhobacteria had no effect on flower senescence in comparison with control (Nayani et al., 1998). In contrast, the dissecting petals of cut flowers in carnations and lychnis before treatment with bacteria caused about 5–6 and 2–3 days delay in senescence, respectively. It is concluded in this way bacterial cells should be taken up within the cut flowers (Glick, 2014). Therefore, the application of endophytes bacteria containing ACC deaminase could be a secure alternative. In this context, Ali et al. (2012) confirmed that the stem colonization of the cut flowers with endophytic strains (*Pseudomonas fluorescens* YsS6 and *Pseudomonas migulae* 8R6) delayed flower senescence about 2–3 days by lowering the ethylene concentration of flower. Thus, use of endophytes bacteria with positive for induction of ACC deaminase activity maintain cut flowers in commercial scales.

21.4.5 Rooting of cuttings

Mayak et al. (1999) treated mung bean cutting plants with ACC deaminase producing bacterium (*P. putida* GR12-2) and its mutant negative for ACC deaminase activity. According to the results obtained, the roots number was similar in the both treatments, while the length of roots was greater in cuttings treatment with ACC deaminase positive strain. In another study, Li et al. (2005) inoculated the carnation cuttings with *Azospirillum brasilense* producing ACC deaminase bacterium. The number and length of roots in inoculated cuttings was more than untreated control cuttings. Montero-Calasanz et al. (2013) tested the efficiency of five bacterial strains in the rooting of olive (*Olea europaea*) cuttings.

Their results showed that the effectiveness of *Pantoea* spp. AG9 strain (positive for ACC deaminase) was more than other strains in the rooting of cuttings.

21.4.6 Metals

Some trace elements, including heavy metals, such as Zn, Cu, Co, Ni and Mn are necessary micronutrients for plant growth, while others such as Cd, Pb, and Hg have an unknown biological function (Asgari Lajayer et al., 2017a, 2019a). Excessive level of heavy metal pollution in soil (due to oxidative stress induction, and their replacement with essential metals in pigments and enzymes) has a harmful effect on biological systems (Asgari Lajayer et al., 2017b, 2019b). Also, high bioavailability of heavy metals restricts the plant growth and the biodiversity (Ghorbanpour et al., 2016). Heavy metals are not biodegradable and can be transformed, immobilized and/or volatilized, but organic pollutants are biodegradable (Shilev et al., 2012). Several traditional approaches such as soil removing, chemical and physical extraction are used for soil remediation (Glick 2014). In another way, the plants employed for remediation of soil from heavy metal, named as a phytoremediation technique. This technique is an environmental-friendly and economically cost-effective way, compare to traditional approaches. Phytoremediation is performed in three ways including phytostabilization (reducing the heavy metals bioavailability and subsequently their stabilization in soil), phytoextraction (metals extraction, transport and accumulation in plant tissues) and phytovolatilization (metals transformation into volatile forms) (Asgari Lajayer et al., 2017a, 2019a). Generally, phytoextraction is used as a preferred way for cleaning up metal pollutants from environment. However, the plants involved in phytoextraction have small size and slow growth, thereby practically use of these plants is limited (Khan et al., 2000). Therefore, the plants employed in successfully soil remediation are characteristic based on one or multiple heavy metal tolerance, highly competitive, fast grow and high biomass production (Glick, 2014). Also, phytoextraction process can be improved by using the PGPRs (Glick, 2010). Belimov et al. (2005) reported that there is a positive correlation between the root elongation of *Brassica juncea* plants and ACC deaminase activity of Cd-tolerant PGPR. Han et al. (2015) inoculated the rice seedling with ACC deaminase containing *Pseudomonas stutzeri* A1501 (wild-type) and A1815 (negative for ACC deaminase activity) under 0.3 mM heavy metal stress. They reported that plants inoculated with *P. stutzeri* A1501 under 0.3 mM heavy metal (Co^{2+} , Cu^{2+} , Zn^{2+} , or Ni^{2+}) stress increased the plant height (19%–30%), root length (39%–47%), root fresh weight (54%–61%), and root dry weight (32%–39%) compared with control plants, however, significant differences were not observed between mutant A1815 strain treated plants and controls. Grobelak et al. (2018) were conducted an experiment for evaluating the potential of ACC deaminase containing bacteria (endophytes and rhizobacteria) in improving plant growth under heavy metal stress conditions. The strains with the highest ACC deaminase activity (mainly belonging to genus *Bacillus* and *Pseudomonas*) were used. It has been shown that inoculation of plants (*Festuca rubra* L. and *Brassica napus* L.) with ACC producing bacteria had a positive effect on growth parameters such as root and stem weight and root and stem length under stress conditions. Also, the activity of ACC deaminase was higher in entophytes strains, which resulted in a higher biomass production of inoculated plants compared to rhizospheric strains treated plants.

21.4.7 Organics

With growing industrialization, environmental problems such as organic pollution (petroleum) areas and saline-alkali soils have become a global concern (Ogri 2001; Sakai et al., 2004; Mohawesh et al., 2014; Sharma et al., 2012). Petroleum is toxic to living organisms, and developing methods for remediation soils polluted by petroleum is necessary. Conventional methods for cleanup soil are time consuming and labor and cost intensive. Furthermore, soil characteristics such as structure and ecology may be destroyed in this way (Merkl et al., 2006; Conesa et al., 2014). In the contrary, the remediation of soil, water, and sediments by plants (phytoremediation) is a cost-effective technique, without any negatively effect on soil structure and ecology (Marinescu et al., 2010). In this technique, plants by absorbing, accumulating and detoxifying the organic pollutants cleaned up the soils contaminated with petroleum (Gerhardt et al., 2009; Ma et al., 2009). However, in phytoremediation process the pollutants inhibit plants growth. An alternative strategy is employing plants and rhizobacteria named as rhizoremediation. In this context, the bacteria are selected based on ability to: (i) IAA synthesis; (ii) degrade soil contaminants; (iii) ACC deaminase production; and (iv) colonize the tissues inside of plants (especially by endophytes bacteria) (Glick, 2014). In a study, the inoculation of *Zea mays* plants by ACC deaminase producing *Gordonia* sp. S2RP-17 strain was significantly improved the growth of plants in the diesel polluted soil compared to control plants (Hong et al., 2011). In another study, oat plants were inoculated with two PGPRs containing ACC deaminase, *Pseudomonas aeruginosa* SLC-2 ($2.52 \pm 0.03 \mu\text{mol } \alpha\text{-KA mg Pr.h}^{-1}$) and *Serratia*

marcescens BC-3 ($38.52 \pm 0.37 \mu\text{mol } \alpha\text{-KA mg Pr.h}^{-1}$) under petroleum pollutant stress (Liu et al., 2015). The results showed that the height and fresh weight of stems was increased (by 23.64 and 42.57%) and (by 16.98 and 28.3%), respectively, whereas the length and fresh weight of roots was increased (by 10.34 and 20.84%) and (24.13 and 43.11%), respectively, when inoculated with SLC-2 and BC-3 compared to non-inoculated controls. The findings indicated that the use of ACC deaminase containing rhizobacteria can serve as promising strategy for improving the phytoremediation efficiency in petroleum-polluted soil. Furthermore, microbial degradation of pollutants is the other strategy for soil remediation. In this regard, ACC deaminase containing rhizobacteria are the most active and have the important potentials (Brooijmans et al., 2009). Therefore, rhizobacteria by degradation of these pollutants may reduce their contents for exposed plants and improve the plant growth (Huang et al., 2004). Liu et al. (2015) reported that the potential of the petroleum degradations of two ACC deaminase containing strains SLC-2 and BC-3 significantly ($P < 0.05$) increased 4.78 and 7.36% respectively, compared to the control.

21.4.8 Pathogens

Plants respond to different pathogens such as bacteria, fungi and nematodes infection/attack by increasing internal ethylene levels (Van Loon et al., 2006). However, seedling inoculation with ACC deaminase containing bacteria may reduce the damage of induced-ethylene by different pathogens, for example, soil-borne plant disease caused by bacteria such as *Pseudomonas syringae* pv tomato (Indiragandhi et al., 2008), *Agrobacterium tumefaciens* (Toklikishvili et al., 2010), *Erwinia* spp. (Wang et al., 2000), and those caused by fungi including *Pythium ultimum* (Wang et al., 2000), *Pythium aphanidermatum* (El-Tarabily, 2013), and *Pyricularia oryzae* (Amutharaj et al., 2012). Also, the efficiency of ACC deaminase containing bacteria has been documented in suppression of the pathogenic nematode caused pine wilt disease like *Bursaphelenchus xylophilus* (Nascimento et al., 2013). Thus, the inoculation of plants with ACC deaminase containing bacteria, as a key trait, protects plants against nematode stress.

21.5 Engineering bacteria and plants

Genetic manipulation of many characteristics from various organisms into a single organism indicates the efficiency of biotechnological techniques. Biotechnology is used for achieving the specific goals and objectives. In this regard, scientists transform *acdS* genes responsible for ACC deaminase expression from microorganisms specially bacteria into the other bacterium or plants. A major focus has been done on rhizobia strains and model plants such as *Arabidopsis*, tomato and canola.

It has been reported that rhizobia species producing ACC deaminase can promote nodulation of their host legumes (Ma et al., 2003a, 2004; Uchiumi et al., 2004), since they decrease ethylene levels that are known to inhibit nodule formation in many leguminous species (Guinel and Geil, 2002; Gage, 2004). For example, *Mesorhizobium ciceri*, the chickpea (*Cicer arietinum* L.) microsymbiont, has no ACC deaminase activity under free-living conditions (Ma et al., 2003b). Conforte et al. (2010) showed that transforming of ACC deaminase in *M. loti* MAFF303099 strain improved nodulation efficiency and competitiveness of this strain in *Lotus* spp. This result demonstrates that the use of *Mesorhizobium* engineered strains containing ACC deaminase activity can be a useful tool to promote nodulation and thus plant growth under normal and stress conditions. In other study, Nascimento et al. (2012) showed that transforming *acdS* gene of *P. putida* UW4, to the LMS-1 (pRKACC) strain caused an increased in nodule formation (by 127%) and total biomass (by 125%) of chickpea plants compared to the plants inoculated with the LMS-1 wild-type strain. Also, Ma et al. (2004) showed that the transformation of an exogenous *acdS* gene into the *Sinorhizobium meliloti* increased its ability to nodulate alfalfa by 35%–40%.

ACC synthase (ACS) is one of the key enzymes in the synthesis of ethylene, and the efficiency of this enzyme in ACC and subsequently ethylene synthesis was documented under stress conditions (Grichko and Glick 2001a). It has been suggested the ACS synthesis in *Arabidopsis thaliana* was regulated with ACS2, ACS6, ACS7, and ACS9 genes (Peng et al., 2005). Therefore, ethylene biosynthesis under stresses such as water logging and salinity reduces plant yield and development (Vanderstraeten and Van Der Straeten, 2017). Recently, the amelioration of stress ethylene within plant tissues for achievement the better yield under stress conditions received more attention. However, there are two pathways for the alleviation of negative effects of stress ethylene on plants: (1) transgenic plants, (2) inoculation by ACC deaminase producing bacteria (Vanderstraeten and Van Der Straeten, 2017; Nascimento et al., 2018). The application of ACC deaminase-containing bacteria as inoculum under different stress conditions such as flooding (Grichko and Glick, 2001a) and high salt concentration (Mayak et al., 2004) have proven with significantly noticeable results. Additionally, several studies showed that the transgenic plants by the ACC deaminase expression mitigated the adverse

effects of environmental stresses by controlling ethylene synthesis (Grichko et al., 2000; Grichko and Glick, 2001b; Sergeeva et al., 2006; Zhang et al., 2015). Grichko et al. (2005) transformed tomato (*Lycopersicon esculentum*) cv. Heinz 902 plants by expressing bacterial ACC deaminase, and tested the effects of metals (Cd, Cu, Co, Mg, Ni, Pb, or Zn) on growth of transgenic tomato plants. Based on expression of ACC deaminase in transgenic tomato plants, a lot of metals accumulated on the tissues of plants. Nie et al. (2002) tested the growth of transgenic canola plants with expressed ACC deaminase genes under arsenate stress. Also, these researchers tested the potential of *Enterobacter cloacae* CAL2 on growth of transgenic and non-transgenic canola plants. According to their results, the accumulation of arsenate in transgenic canola plants was more than control. Similarly, Jung et al. (2018) tested the growth and development of transgenic *A. thaliana* (line S11 and S14 strong and weak expression of ACC deaminase) under water logging and salt stresses. The high levels of stress tolerance were observed in transgenic plants especially in line S11 followed by S14, compared to the control plants.

Also, Farwell et al. (2006) stated that application of transgenic canola and *P. putida* can improve phytoremediation of nickel in soil compared with un-inoculated plants. In other study Farwell et al. (2007) tested the growth of transgenic canola and no transformed canola inoculated with *P. putida* containing ACC deaminase activity under flooding and metal stress. The results showed that the growth of canola was decreased under flooding condition, while the accumulation of nickel was increased in both transgenic and non-transgenic plant tissues. Therefore, utilization of transgenic plants containing ACC deaminase genes and the inoculation by PGPR containing ACC deaminase are two promising strategies in management of stressed agriculture.

Similar to ACC deaminases producing bacteria and the transgenic plants by ACC deaminase expression activity, endophytic ACC deaminase containing bacteria may ameliorate the negative effects of ethylene on plants. Also, this ability of bacteria can be used to study the metabolic activities engineered in plants (Duan et al., 2009). In this regard, Liu et al. (2017) designed a protocol for constructing engineered strains with cell surface expression of ACC deaminase gene on two endophytic bacteria (*Enterobacter* sp. E5P and *Kosakonia* sp. S1P). This experiment was based on hypothesis that engineered endophytic bacteria affect the metabolic activities such as concentration of ethylene and stress tolerance in rice plants compared with wild strains (*Enterobacter* sp. E5 and *Kosakonia* sp. S1). They showed that ACC deaminase activity of the engineered strains was significantly higher than the wild strains, indicating ACC deaminase is intercellular enzyme. Also, the disruption of cells by toluene resulted that the ACC deaminase expression on the cell surface of E5P and S1P was about 41 and 32%, respectively. Similarly, ACC deaminase activity in rice seedling inoculated by endophytic bacteria was increased compared to controls. However, plants inoculated by engineered strains showed higher activity of ACC deaminase (a cytoplasmic enzyme) about 15 and 21% than wild strains. Since ACC is readily diffused in water it can easily be absorbed by bacteria, and can ultimately degrade it. Therefore, in engineered strains the ACC deaminase directly could contact with ACC excluded from plants. Subsequently, these bacteria can reduce the ethylene levels of plant tissues by cell surface expression of ACC deaminase. Also, efficiency of the engineered strains was tested on rice growth under 1, 1.5, 2 and 2.5% saline stress. The results showed that the growth parameters such as germination rate, root and shoot length, fresh and dry weights of roots and shoots were improved in inoculated plants with E5 and S1 compared with the controls. However, the efficiency of engineered strains on growth parameters were more pronounced than the wild types. Therefore, the utilization of engineered endophytic strains could be considered as an effective strategy to improve plants growth under stressful conditions.

21.6 Conclusions and future prospects

Agricultural productivity is reduced due to environmental stresses such as salinity, drought, pH, temperature, heavy metals, organic pollutants, water logging and phytopathogens. The effect of these stressors in reduction of growth for the most crop plants is estimated to be more than 50%. Therefore, global crop production needs to double for feeding the world population. Apart from this problem, plant productivity is negatively affected by stress ethylene. Recently, the use of plant growth-promoting bacteria as an efficient tool for agricultural productivity has attracted considerable attention. These bacteria through the synthesis of ACC deaminase can reduce the adverse effects of ethylene production under stressful environments. The efficiency of ACC deaminase producing bacteria have been well documented under biotic and abiotic stresses by researchers. The ACC deaminase gene (*acdS*) is widespread in different bacterial species. Studies showed that the abundance of *acdS* gene is affected by adverse conditions and plant genotype. Therefore, it is concluded that the isolation of ACC deaminase producing bacteria from stressed conditions and the rhizosphere of resistance plants can successfully improve the plant growth under environmental stresses. Up to date, in biotechnology the gene *acdS* is transformed in rhizobia strains and model plants such as *Arabidopsis*, tomato and canola. Compare to free living bacteria, ACC deaminase activity in N2 fixer bacteria is very low. Also, some of rhizobia strains have no ACC

deaminase activity in free living conditions. Therefore, by transforming gene *acdS* in these strains can improve their efficiency in root colonization and nodulation performance. The efficiency of transgenic plants was proven in several studies. However, this technique employs only model plants and should be tested on many field crop plants. More recently, the improvement of endophytic bacterial ACC deaminase received more attention. In this regard, ACC deaminase was located on cell surface and more active than cytoplasmic ACC deaminase. However, further researches should be performed on other endophytic bacteria and the efficiency of engineered bacteria test on many crop plants. It is hoped that the biotechnology techniques by increasing agricultural productivity will help to feed all of the world's population in the future.

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Chapter 22

Role of rhizobacteria in alleviating salt stress

S. Srividhya¹, Nidhi Kumari², R. Surendranath³ and P. Jeyakumar⁴

¹Division of Field Crop Improvement and Protection, ICAR- Central Island Agricultural Research Institute, Port Blair, India, ²Division of Crop Protection, ICAR- Central Institute of Subtropical Horticulture, Lucknow, India, ³Department of Horticulture and Sericulture, Hyderabad, India, ⁴Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore, India

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22.1 Introduction

Salinity is one of the major stresses affecting plant growth and development next only to drought and heat stress. Due to expanding climate change effects, especially in the arid and semi-arid regions, area under salinity has increased to about 397 million ha (saline soils) and 434 million ha (sodic soils) globally. The FAO/UNESCO soil map estimates reveal that 19.5% (45 million ha) of total irrigated land and 2.1% (32 million) of the dryland agriculture is affected by salinity. Although, accumulation of salts is a natural process, the fraction of salt affected area is constantly increasing due to climate change conditions (Othman et al., 2006) such as droughts, sea level rise and flash floods, etc. Apart from these factors, excess use of chemical fertilizers and leaching of salts in the irrigated lands affect the crop growth and productivity. Hence, long term salinization ultimately alters the soil properties and fertility, reduces microbiota functions, affecting the crop growth and development (Munns and Tester, 2008; Fukhran, 2016).

Soil salinity occurs when the concentration of toxic salts exceeds the threshold having (a) EC more than 4 dS m⁻¹ (b) Exchangeable Sodium Percentage (ESP) less than 15 and (c) pH less than 8.5. These saline soils are characterized by the white crusts over the soil surface. The salts causing this are due to the presence of sodium, calcium, magnesium, chlorine, sulfate, and even nitrate. Non-saline alkali soils are categorized by that which include (d) EC less than 4 dS m⁻¹ (e) Exchangeable Sodium Percentage (ESP) more than 15 and (f) pH more than 8.5. On the other hand, saline-alkaline soils are characterized by the presence of (g) EC more than 4 dS m⁻¹ (h) Exchangeable Sodium Percentage (ESP) more than 15 and (i) pH usually less than 8.5 with higher content of sodium. These soils are formed as a result of salinization and alkalization.

Most of the cultivated crops have lesser threshold/tolerance to salt stress. Thus, the primary and secondary cause of soil salinity has a huge impact on limiting crop yields. Poor irrigation practices, poor drainage in the soil combined with erratic rainfall restricts most plant growth, due to the high osmotic pressure of the soil solution that makes water uptake by plants difficult. In a recent meeting held by the Global framework on Water Scarcity in Agriculture (WASAG) at FAO in 2018, proposed that up-scaling of climate-smart agricultural practices and new remediation methods have to be adopted for the management of salt affected soils.

The present chapters henceforth will discuss elaborately salinity response in plants, the hormonal interactions and then the vital role of rhizobacteria (an ecofriendly approach) in alleviating salt stress in crops. Plant Growth Promoting Rhizobacterias' (PGPRs) are promising and potential soil microorganisms that enhance plant health and promote plant growth under stressful conditions (Glick, 2012; Lugtenberg et al., 2013; Vejan et al., 2016). The term "Plant Growth Promoting Rhizobacteria (PGPR)" was used to denote the beneficial microbes present in the rhizosphere by Kloepper and Schroth (1978). In the recent era of climate change, PGPRs is one of the most viable solutions for combating abiotic stresses and sustainable agriculture (Glick et al., 2007; Dodd and Pérez-Alfocea, 2012; Berg et al., 2013).

22.2 Salinity response in plants

Plants undergo various responses to tolerate the effects of salt stress. Crop plants differ greatly in their tolerance to genetic variation, as revealed by their growth responses. The salts accumulate in the root zones and restrict nutrient uptake. Two major phases are shown by salinity stressed plant: (i) osmotic stress (primary and rapid effect) (ii) ionic stress (secondary and slower effect). The excess salts in the soil make the water unavailable to the plants (physiological drought), as there is increase in the osmotic pressure of soil solution. Apart from the osmotic effect, high concentration of ions in the solution and their absorption may prove toxic to the plants. Some of the physiological processes affected at the cellular level, during salt stress are the loss of cell turgor, reduction of cell elongation, lower cell division rates, altered enzyme activities, production of stress phytohormones and production of reactive oxygen species inside the cell. High salinity causes drop in the cytosolic K^+ concentrations inactivating the enzymes and proteins for normal functioning. Evidence to this explained in Cramer et al. (1985) states that high Na^+ ions can alter root permeability as a leakage of K^+ and Ca^{2+} displacement. Saline soil has lower affinity to uptake K^+ . Also, at the cellular level, high salts are accumulated in the vacuole due to compartmentation of ions thus protecting the enzymes. ROS including hydrogen peroxide, superoxide and free oxygen radical causes lipid peroxidation and induces programmed cell death that affects the membrane permeability and plant processes (Apel and Hirt, 2004). Thus, two most significant responses at the cellular level for salt tolerance (i) compartmentation into vacuoles and (ii) osmotic adjustment via osmolytes.

22.2.1 Effect of salinity on plant phenological development

At the whole plant level, lesser seed germination, poor seedling establishment, reduction in leaf expansion, reduced vegetative growth, nutrient imbalance, changes in plant density, early flowering, early maturity, reduced photosynthesis, restricted enzyme activities, decrease in nodule number, nutrient deficiency symptoms and associated plant diseases including premature senescence and ultimately yield loss with affect in produce quality are perturbed under salt stress. Atak et al. (2006) and Neamatollahi et al. (2009) reported that higher salt concentrations may decrease the germination percentage due to increased osmotic pressure. All these processes affected by salt stress directly or indirectly limit the crop yield and productivity. Some mechanisms include the synthesis of compatible solutes in the cell, antioxidants, phytohormones, activation of enzymes like ACC deaminase and certain secondary metabolites help crops to tolerate the salinity effects to a larger extent (Munns, 2002a).

Several studies have confirmed that salinity inhibits seed germination of crops such as wheat (Egamberdieva, 2009), rice (Xu et al., 2011), maize (Khodarahmpour et al., 2012), and soybean (Essa, 2002), faba bean (Rabie and Almadini, 2005). Also, Jamil et al. (2006) observed significant reductions in germination percentage, germination rate, and in seedling root and shoot lengths of cabbage, sugar beet, paniculate amaranthus, and pak-choi. In another study by Egamberdieva (2011), increasing the salt content reduced the shoot length (50%) and root length (7%) of bean seedling grown in a gnotobiotic sand system in a growth cabinet.

Munns (2002b), reported that genetic traits and environmental adaptations regulate salinity tolerance mechanisms in glycophytes and halophytes. Halophytes, that possess tolerance to high salinity levels, carry salts through the xylem and store them in the leaves. These plants have specialized adaptations such as 'salt glands' in the shoots to excrete the excess salt, which can be removed by the wind or water. Halophytes are said to have very low ψ_s (solute potential) enough to lower ψ_w (plant water potential), that enables to imbibe more water that limits excess salt absorption

(Taiz and Zeiger, 2010) from the dissolved solutes. Some of the plants like *Suaeda maritima* and *Atriplex nummularia* have tolerance to salt concentrations much above the threshold level of glycophytes.

On the contrary, glycophytes (salt sensitive) avoids the uptake of salts through the root membrane (Taiz and Zeiger, 2010). Glycophytes, also termed non-halophytes are sensitive to salt concentrations and cannot tolerate salts unlike halophytes. Above the threshold concentrations, most of the cultivated crops that fall under the category of glycophytes have poor ion partitioning at the cellular level (Lauchli and Epstein, 1990) and also in the shoots. The energy driven symplastic movement of salt ions like Na^+ could reduce the accumulation in shoots. When the salt concentration exceeds the rate of exclusion by roots or cellular ability to compartmentalize salts in the vacuoles, it builds up in the cytosol, disrupts cellular structures and functions (Stewart and Ahmad, 1983; Munns, 2002b; Munns, 2005; Roy et al., 2014).

In general, threshold level of salt tolerance of crop species varies from 40 to 200 mM NaCl although all plants are sensitive to salt at one particular concentration. Accumulation of salts impairs plant functions such as photosynthesis, protein and lipid metabolism, nitrate assimilation, membrane leakage, reduction in chlorophyll pigments (Ball and Sobrado, 1999) and enzyme activities like nitrogenase (Rai and Tiwari, 1999). But most plant follow suit a series of mechanisms that enable them to sustain growth. Salt stress induces a phenomenon called salt respiration at the rhizosphere in the glycophytes for excluding excess Na^+ , and Cl^- . The crop yield reduction owing to salt stress could be attributed to the larger modification at the cellular level (like shrinkage in cell contents, membrane injury, etc.). Ion selectivity regulated by Ca^{2+} and K^+ ions regulated Na^+ concentrations inside the cell (Zhong and Lauchli, 1994; Liu and Zhu, 1997; Zhu, 2001). Salt stress tolerance in plants is therefore directed towards maintaining osmotic balance and ion homeostasis by different mechanisms (Munns and Tester, 2008; Ilangumaran and Smith, 2017). Salinity most importantly is detrimental to the symbiotic association between legumes and Rhizobium bacteria below ground posing deleterious effects.

Root elongation rate recovers after initial exposure to salt but the root architecture is altered where, high salt concentration represses formation of lateral roots. Alterations in the root architecture, reduction in population of beneficial microbes bring about a nutrient imbalance under saline stress. High concentrations of sodium ions at the root zone intrude the uptake of potassium ions. Continuous salt stress on the plants can further lead to a reduction in accessibility and uptake of some elements such as N, P, K, and Mg due to abundance of exchangeable Na^+ and Cl^- ions (Heidari and Jamshid, 2010). Besides, it has also been reported that excess sodium chloride concentration in the shoot causes reduction in photosynthesis to its half without affecting the concentration of chlorophyll (Dionisio-Sese and Tobita, 2000). All the above discussed processes overlap to that of drought stress, bring about a huge impact on the photosynthesis, including leaf senescence, yield loss with significant growth impairment (Munns, 2002a).

22.2.2 Activation of signal transduction and hormonal regulation in plants under salt stress

Activation of the signaling molecules and stress responsive genes is an important regulatory mechanism under any stress condition. Hormonal regulation under salt stress is another important mechanism modulating salt stress response and tolerance. Under salt stress, activation of SOS 1, 2 and 3 (Salt overly Sensitive) genes for ion homeostasis; activation of Ca^{2+} sensor proteins followed by activation of TFs (MAPK, DREB, NAC, WRKY, and bZIP); up-regulation of genes (P5CS, Aquaporin, ABA responsive LEA GENES, NCED, photosynthetic genes) coordinated salt tolerance in plants.

Phytohormone signaling cascades influence the salt tolerance mechanisms (Ilangumaran and Smith, 2017) and regulate plant adaptation to salinity (Waśkiewicz et al., 2016). During the different plant phenophase, plant induces synthesis of hormones like Auxin (IAA), Cytokinin, ABA (abscisic acid), GA (Gibberellic acid), SA (Salicylic acid), Br (Brassinolide), PA (Polyamines), JA (Jasmonic acid) and ethylene (Orsini et al., 2010; Ryu and Cho, 2015; Muchate et al., 2016). Soil salinity also induces ABA synthesis in the leaf, which leads to stomatal closure, and reduced photosynthesis. An immediate response of salinity on plant development is inhibition of cell growth through ABA synthesis (Das et al., 2015).

22.2.3 Exploiting plant growth promoting rhizobacteria (pgprs) for mitigating salt stress

The efforts to breed for salt tolerant crops have attained only limited success due to the soil heterogeneity, polygenic and physiological complexity of the salt tolerance trait (Araus et al., 2008; Dwivedi et al., 2010). The recent promising and alternative approach to ameliorate/mitigate salt stress could be the use of beneficial microbes (Lugtenberg et al., 2013; Berg et al., 2013; Egamberdieva and Lugtenberg, 2014) that have the ability to promote and sustain growth.

Several studies have reviewed and proven the beneficial and significant influence of PGPRs on plant growth and development (Lau and Lennon, 2011; Bhattacharyya and Jha, 2012). However, the commercial practice or employment of PGPRs for inducing salt stress tolerance at a global level is definitely negligible, as large data is confined to the research experiments.

Soil microbes (PGPRs) colonizing the rhizosphere are highly beneficial and can also suppress the pathogenic microbes inhabiting the root zone (Egamberdieva and Lugtenberg, 2014) and help control diseases. The effect of PGPR as biocontrol agents against harmful microbes is possibly due to several mechanisms like production of cell wall degrading enzymes, induced systemic resistance and better nutrient availability (Kotan et al., 2009; Bhattacharyya and Jha, 2012). Among the diversity of microorganisms at the rhizosphere, the beneficial bacteria's are known to greatly influence the plant growth, aid in nutrient acquisition and induce signals to tolerate biotic/abiotic stress factors. In general, PGPR inoculation affects under salt stress may encompasses several mechanisms including increase in root surface area, regulation of plant endogenous hormones, nutrient solubilization, synthesizing organic acids, ACC-deaminase, gulcanase and chitinases, etc (Glick et al., 2007; Berg, 2009; Hayat et al., 2010). The exact cause of growth promotion under salt stress by the PGPR is difficult to be defined as it may arise via direct or indirect mechanisms. The beneficial aspects of a range of PGPRs is represented in Table 22.1, the mechanism by which they induce stress tolerance is depicted in Fig. 22.1.

The beneficial microorganisms belong to several genera such as *Rhizobium*, *Bradyrhizobium*, *azotobacter*, *azospirillum*, *Pseudomonas*, *Burkholderia*, *Achromobacter*, *Methylobacterium* and *Bacillus* (Grover et al., 2011; Vejan et al., 2016; Van Oosten et al., 2017). In fact, these have been broadly termed as PGPM which includes (i) arbuscular mycorrhizal fungi (AMF) (Jeffries et al., 2003), Plant Growth Promoting Rhizobacteria (PGPR) (Podile and Kishore, 2006), and nitrogen fixing rhizobia (Franche et al., 2009). The tolerance level of PGPR varies from 100 to 650 mM of NaCl, making its suitability for application in inducing salt tolerance of plants. Most of the crop species are highly susceptible to salt stress with concentration of dissolved ions exceeding $> 60\text{--}80\text{ mM}$ and $\text{EC} > 5.0\text{ ds m}^{-1}$ (Horneck et al., 2007; Ondrasek et al., 2009). It is also reported that several PGPR strain are saline tolerant ($> 3\%$ NaCl) and thermostable (up to 40°C) due to their efficacy (Yildirim et al., 2008; Nadeem et al., 2010a; Yao et al., 2010; Marulanda et al., 2010; Yildirim et al., 2011; El-Akhal et al., 2013; Ahmad et al., 2013a,b). The microbial genus consisting of *Beggiota*, *Thiomargarita*, *Thioplota* and *Bacillus* sp. have the ability to sequester extra nitrate into the vacuoles, also protecting the plant from nutrient toxicities under stress conditions (Chaudhuri et al., 2017). Also, PGPR is also an alternative way for bioremediation that could remove hydrocarbons, some of the heavy metals, etc.

22.2.4 Selection and functional characterization of PGPR strains

Potential and stable strains isolated from several crop rhizosphere/endorhizosphere zones must be evaluated for their mechanism of action. This involves simultaneous purification, selection under gnotobiotic conditions, ability to synthesize phytohormones like auxin, release of volatiles, etc. (Saharan and Nehra, 2011). This leads to identification of strains that could exhibit one or many mechanisms to induce response under saline conditions (Joseph et al., 2007) and other stresses. After this process, product development as biofertilizers, bioagents, biostimulants or bioprotectants must be designed to suit the need and all the environmental conditions.

An experiment conducted by Cordovilla et al. (1999a,b) to study the performance of two different *Rhizobium leguminosarum* strains on legumes (pea and fava bean) depicted that the salt tolerant GRA19 strain performed better in mitigating moderate salt stress compared to salt sensitive (GRL19). The legume plants were observed to have larger nodules and higher N_2 fixation even under salinity stress. Thus, tolerance to a particular stress varies within different strains of a PGPR (Van Oosten et al., 2017). Certain strains of *Rhizobium leguminosarum* (SAAN1) can withstand salt concentrations up to 0.34 M NaCl, but with fewer nodules in saline soils (Suarez et al., 2015). In barley, E19 (T) strain of *H. diatomophila* inoculation under salt stress, improved root colonization and also recorded significant increase in root and shoot biomass, with higher RWC in the tissues. The changes in root exudate composition like root phospholipid, ATPase, organic acid content might have resulted in increased tolerance to salt stress (Pereyra et al., 2006).

22.2.5 Consortia effect

PGPR applied in combination with one or more sp. (microbial consortia) have also shown greater tolerance under stress conditions. Inoculation of *A. brasilense* and *Pantoea dispersa* in sweet pepper plants showed higher rates of photosynthesis, more dry matter under salt stress without any effect on PS II efficiency and chlorophyll concentration. Another study conducted by Egamberdieva et al. (2016), also indicated efficacy of combined inoculation of *P. extromoreintalis*

TABLE 22.1 Current perspective of PGPRs exploited for alleviating salt stress in plants.

PGPR applied as a single strain or co-inoculation of strains	Crops	Direct/indirect mode/ mechanisms of action in mitigating salt stress	Salt tolerance level	References
<i>Bradyrhizobium japonicum</i>	Soybean	Enhanced plant growth and yield; increase in nodulation and protein levels, improved N and P uptake with improvement in the root architecture and associated traits	61 to 75 mM NaCl; 5.0 dS m ⁻¹	Han and Lee, 2005; Miransari and Smith, 2008; Egamberdieva et al., 2016
<i>Streptomyces venezuelae</i> ATC	Thai rice	Decrease in the levels of ethylene, reactive oxygen species (ROS) and Na ⁺ , while increased levels of proline, total chlorophyll, relative water content (RWC), malondialdehyde (MDA) and K ⁺ uptake	150 mM NaCl	Yoolong et al., 2019
<i>Bacillus megaterium</i>	Maize	Improved root hydraulic conductance	150 mM NaCl	Marulanda et al., 2010
<i>Pseudomonas putida</i> 108, <i>Pseudomonas fluorescens</i> 153, <i>Pseudomonas chlororaphis</i> TSAU13, <i>Pseudomonas extremorientalis</i> TSAU20	Wheat, Bean, Cotton	Increased plant growth, shoot growth, dry weight, stimulation of root growth, high grain yield and 1000 g seed weight	12 dS m ⁻¹	Abbaspoor et al., 2009; Yao et al., 2010; Egamberdieva, 2011; Egamberdieva and Jabbarova, 2013
			7.1 dS m ⁻¹	
<i>Azospirillum brasilense</i> NH	Wheat	Improved germination and growth	200 mM NaCl	Rabie and Almadini, 2005; Dardanelli et al., 2008; Nabti et al., 2010
	Faba bean	Root branching was promoted, enhanced secretion of flavonoids and LCOs, nitrogenase enzymes and higher NPK uptake,	6 dS m ⁻¹	
<i>Pantoea agglomerans</i>	Maize	Upregulation of aquaporin gene PIP 2–1 gene and increased biomass	0.2 mol l ⁻¹ NaCl	Gond et al., 2015
<i>Pseudomonas pusedoalcaligenes</i>	Rice	Synthesis of compatible osmolytes like glycine betaine, higher shoot biomass	2.5 g NaCl kg ⁻¹ soil	Jha et al., 2011
<i>Bacillus subtilis</i> GB03	Arabidopsis	Induced systemic resistance by production of VOCs, ion homeostasis, up-regulation of HKT1 in shoots	100 mM NaCl	Zhang et al., 2008
<i>Pseudomonas simiae</i>	Soybean	Synthesis of VOCs, proline, Na ⁺ homeostasis, increased chlorophyll content	100 mM NaCl	Vaishnav et al., 2015
<i>Pseudomonas putida</i> AKMP7	Maize	Decrease in electrolyte leakage, osmoregulant production and maintenance of leaf water potential with selective uptake of K ⁺ ions, ACC deaminase synthesis	100 mM NaCl	Bano and Fatima, 2009
<i>Pseudomonas putida</i> UW4	Canola		100 mM NaCl	Cheng et al., 2007
<i>Sinorhizobium mellilote</i> R29, R103, <i>Rhizobium leguminosarum</i> bv. <i>Phaseoli</i> strains R281, R307	Canola	ACC deaminase producing halotolerant PGPR strains increased the shoot and root growth, dry weight, increased the RWC, improved the salt tolerant index	50 mM NaCl + MgCl ₂	Saghafi et al., 2019

(Continued)

TABLE 22.1 (Continued)

PGPR applied as a single strain or co-inoculation of strains	Crops	Direct/indirect mode/mechanisms of action in mitigating salt stress	Salt tolerance level	References
<i>Pseudomonas frederiksbergensis</i> OS261	Red pepper	Increased plant height, reduced ethylene emission, increased dry biomass and showed enhanced antioxidant enzyme activity in leaf tissues	Up to 150 mM of NaCl	Chatterjee et al., 2017
<i>Rhizobium</i> (strain Thal-8) and phosphate-solubilizing bacteria (<i>Pseudomonas</i> sp. 54RB) applied alone and in combination	Maize	Decrease in electrolyte leakage, osmoregulant production and maintenance of leaf water potential with selective uptake of K ⁺ ions	100 mM NaCl	Bano and Fatima, 2009
<i>Achromobacter piechaudii</i> ARV8	Tomato	Increase in the seedling fresh and dry weight, slightly increase in phosphorous and potassium uptake, improved WUE	172 mM NaCl	Mayak et al., 2004
<i>Streptomyces</i> sp. strain PGPA39	'Micro Tom' tomato	Synthesis of ACC deaminase and IAA, improved uptake of phosphorus with increase in the number of lateral roots and biomass, higher chlorophyll content but with lesser proline levels	180 mM NaCl	Palaniyandi et al., 2014
Co-inoculation of <i>Rhizobium phaseoli</i> & <i>P. fluorescens</i>	Mungbean	Overall improvement in the growth, better ionic balance, increase in the water use efficiency, phosphorus uptake, higher photosynthetic rate	ECe = 4.1–6.7 dS m ⁻¹	Ahmad et al., 2013a,b
<i>Bacillus amyloliquefaciens</i> NBRISN13	Rice	↑↑ of salt stress response genes like SOS1, EREBP, SERK1, NADP-Me2	200 mM NaCl	Nautiyal et al., 2013
<i>Bacillus amyloliquefaciens</i> SQR9	Maize	Improved peroxidase and catalase enzyme activity, increased sugar osmolites, chlorophyll content and leaf K ⁺ /Na ⁺ ratio and prevented salinity induced stomatal closure caused by ABA. Also ↑↑ of Rubisco subunit genes, HKT1, NHX1,2,3, H(+)Ppase and ↓↓ of NCED expression	100 mM NaCl	Chen et al., 2016;
<i>Enterobacter</i> sp. UPMR18	Okra	Production of ACC deaminase gene to nullify ethylene induced root inhibition, enhanced antioxidant enzyme activity via ↑↑ of ROS pathway genes	75 mM NaCl	Habib et al., 2016
<i>Enterobacter</i> sp. and <i>P. fluorescens</i>	Maize	Both the strains could synthesis ACC deaminase, improved K ⁺ uptake by Na ⁺ exclusion and NPK uptake with significant improvement in the yield parameters	-	Nadeem et al., 2009
<i>P. fluorescens</i>	Groundnut	Production of ACC deaminase, yield improvement	120 mM NaCl	Saravanakumar and Samiyappan, 2007

(Continued)

TABLE 22.1 (Continued)

PGPR applied as a single strain or co-inoculation of strains	Crops	Direct/indirect mode/mechanisms of action in mitigating salt stress	Salt tolerance level	References
<i>Azospirillum brasilense</i> Sp245 Sp	Lettuce	Improved growth with better produce quality and extended shelf life	40 mM NaCl	Gabriela et al., 2015
<i>Rhizobium leguminosarum</i> vicia strain GRA 19, 3841 and <i>Bradyrhizobium</i> sp.	Faba bean	Improved root nodulation, increased plant biomass and nitrogen content, higher glutamine synthetase	100–120 mM NaCl	Cordovilla et al., 1999a,b; El-Hamdaoui et al., 2003
Co-inoculation of <i>Rhizobium tropici</i> and <i>paenibacillus polymyxa</i>	Phaseolus bean	Increase in the root nodule number, N uptake	-	Figueiredo et al., 2008
<i>Burkholderia</i> sp. P50	Rice	Improved root colonization, reduced ethylene levels by production of ACC deaminase, improvement in plant growth	300mMNaCl	
<i>Azotobacter chroococcum</i> A2	Wheat, pea, rice potato cotton	Significant improvement in the root and shoot growth with higher economic yield	Saline-arid soils	Egamberdiyeva and Höflich, 2003, 2004
<i>Brevibacterium linens</i> RS16	Rice	enhanced photosynthesis, and reduced ACC accumulation, ACC oxidase activity and VOCs, increase in plant physiological activity	100 mM NaCl	Chatterjee et al., 2018

SALINITY STRESS EFFECTS ON PLANTS

- **Early responses** like decrease in plant water potential, inhibition of root growth, cell elongation, membrane leakage, seed germination, leaf development, ROS production, reduced stomatal conductance, low RWC, reduction in leaf CO_2 .
- **Prolonged exposure** causes leaf senescence, ethylene induced root growth inhibition, shoot toxicity due to Na^+ uptake

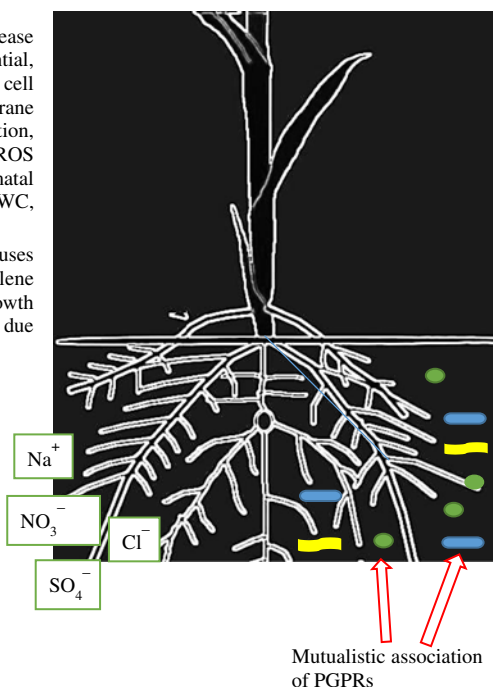


FIGURE 22.1 Effect of salt stress on plants (left) and role of PGPR in mitigating the stress.

EFFECT OF PGPRs IN MITIGATING SALT STRESS TOLERANCE

- **At the cellular and whole plant level** inoculation helps in root colonization, nutrient fixation (symbiotic) and nutrient solubilization for improving uptake and cation exchange capacity, K^+ uptake, production of compatible osmolytes & growth promoting phytohormones, synthesis of siderophores, VOC's and root exudates, ion homeostasis
- PGPRs also synthesize enzymes like LCOs, EPS, PAs for alleviation of oxidative damage by antioxidants, ACC-deaminase to counteract ethylene induced root inhibition,
- **At the gene level**, it upregulates NHX and HKT transporters, H^+ Ppase, SOS genes, NOD-genes, WRKY, DREB, RD29A, LEA genes, P5C5S, Rubisco subunit genes and downregulates the NCED gene, ROS pathway genes and ACC gene

TSAU 20 (root colonizing PGPR) and symbiotic *mesorhizobium* strains mitigated salt stress, with improved nodule development and nitrogen fixation. The mutualistic associations of PGPRs might have enhanced the antioxidant enzyme activity, higher photosynthesis, better nutrient acquisition and plant growth under salt stress conditions (Fig. 22.1).

Co-inoculation of rhizobacterial strains depicted synergism in inducing seed germination and plant growth under salinity stress (Yildirim et al., 2008). A PGPR consortia tested under multiple trials applied to the soil improved the mungbean seed germination, seed vigor with overall increase in plant agronomic parameters (number of branches, plant height, pods/plant, seed yield and weight) as compared to un-inoculated plants (Panwar et al., 2016). PGPR inoculation in the seed may be vertically transmitted (Ahmad et al., 2008). A significant yield increase twice that of the control was also observed along with improved soil fertility (Chaudhuri et al., 2017). Synergistic effects of combined inoculation of two or more PGPRs (*Chryseobacterium balustinum* and *Sinorhizobium fredii*) can help in promoting root growth and nodule development in legumes including soybean (Estevez et al., 2009).

22.2.6 Osmotic adjustment and ion homeostasis by PGPR inoculation

Osmotic adjustment is a metabolic adaptation at the cellular level recruited by PGPR under salinity stress. The various compounds accumulated in the bacterial cell such as proline, glycine betaine, mannitol, sorbitol and sugars such as trehalose (a non-reducing sugar), tetrahydropyrimidines (ectoines) maintain the turgor potential under osmotic stress (Galinski and Trüper, 1994; Bohnert et al., 1995; Thomas et al., 1995; Mäkelä et al., 2000; Ben Ahmed et al., 2010; Ahmad et al., 2013a,b) in plants via the transport system (Arora et al., 2012) and vice versa. These osmolytes do not hinder enzyme functions inside the cell. Executing an effect on the transpiration could be a possible mechanism mediated by PGPR to maintain the water potential in plants (Dodd and Pérez-Alfocea, 2012). Production of poly- β -hydroxyl butyrate (PHB) in rhizobial cells have been reported to be one of the protective measures under toxic salt levels (Arora et al., 2006). The PGPR *Sinorhizobium meliloti* is highly efficient in synthesizing osmolytes.

Ion homeostasis is an important mechanism facilitated by PGPR inoculation for salt tolerance in plants. Salt uptake in plant is inhibited by the PGPRs at the rhizoplane with the excess ions being trapped in the exopolysaccharide secretions (Ilangumaran and Smith, 2017) and by activation of ion transporters. The above ground changes are reduction in Na^+ accumulation in the leaves, high K^+ uptake in the shoots via up-regulation of potassium transporters, Cl^- exclusion in the leaves while the below ground changes are Na^+ exclusion in the roots, siderophores secretion, by modification of rhizosheaths and improved nutrient uptake via up-regulation of SOS pathway genes. Maize plants inoculated with *Azotobacter* sp. was responsible in maintaining ion homeostasis with higher levels on osmolites when grown under saline conditions (Rojas-Tapias et al., 2012). PGPR thus act in improving stress conditions by affecting cell elongation and membrane permeability. Field trials of PGPR application in basil, groundnut and maize revealed enhanced proline content aided with higher chlorophyll levels and water status for salinity tolerance (Saravanakumar and Samiyappan, 2007; Nadeem et al., 2009; Heidari et al., 2011).

22.2.7 Alleviation via phytohormone production and gene regulation

PGPRs activate signaling molecules for phytohormone production and metabolite synthesis in the plants as a result of bioactive metabolites and hormone synthesis by PGPR itself. These molecules not only confer stress tolerance both biotic and abiotic, but also contribute for overall improvement in the allometry. PGPRs like *Lactobacillus* sp. and *A. chroococcum* also have potential roles in altering the root zone by executing quorum sensing compounds under stress conditions (Yildirim et al., 2008; Barriuso et al., 2008; Upadhyay and Singh, 2015).

PGPR inoculation (*Bacillus amyloliquefaciens* SQR9 strain) in vitro enhanced salt stress tolerance in maize seedlings by up regulation of continuum genes including RBCS, RBCL genes, H^+ PPase, HKT1, NHX1, NHX2, NHX3 genes and down regulation of NCED expression (Chen et al., 2016). *Pantoea agglomerans*, stable under hyper saline conditions up-regulated the aquaporin gene (plasma membrane integral protein *PIP 2-1*) while inducing salt tolerance in maize (Gond et al., 2015). Also, aquaporin gene *PIP2* was crucial for salt tolerance in maize plants inoculated with *Bacillus megaterium* (Marulanda et al., 2010) exhibiting better root hydraulic conductance although a basal level expression of the genes was observed in un-inoculated plants. The exact mechanism by which rhizobacteria induce aquaporin expression is not clear though.

The growth promoting bacteria's synthesize and secrete hormones, which gets adsorbed on the seed or the root surface of the plants (Fallik et al., 1994) and mediate tolerance along with the plant endogenous hormones. PGPR inoculation in chickpea (with *Pantoea dispersa*, PSB3) produced IAA (Indole acetic acid) and ACC deaminase that brought about an improvement in plant biomass and yield. The study also documented a reduction in membrane leakage, Na^+

exclusion, K^+ uptake and maintenance of relative water content (RWC) and chlorophyll content (Panwar et al., 2016). PGPR application has a role in inhibiting the salinity induced ABA level thereby regulating stomatal conductance. Inoculation also facilitates K^+ uptake, enhanced accumulation of compatible osmolites, glutathione content, and antioxidant enzyme production in the plants. *Streptomyces* sp. capable to synthesizing IAA and secrete siderophores induced salinity tolerance in wheat plants (Sadeghi et al., 2012). Nadeem et al. (2009) also recorded salt tolerance in maize with combined inoculation of *Pseudomonas fluorescence* and *Enterobacter* sp. strains capable of synthesizing ACC deaminase. PGPR inoculation in lettuce with *Bacillus subtilis* significantly improved the root-to-shoot signaling via cytokinin production (Arkhipova et al., 2007; Dodd et al., 2010) under salt stress conditions. Similarly, PGPRs can increase shoot cytokinin and alleviate salt stress in wheat seedlings when inoculated with *Pseudomonas* strains TSAU22, TSAU6 and TSAU 20. PGPR could also inhibit salt stress affects in capsicum when inoculated with *Arthrobacter* sp. and *Bacillus* sp. which produced IAA and ACC deaminase in the roots (Sziderics et al., 2007).

22.2.8 Mitigating ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase

PGPR has been proven to mitigate ethylene distress in the root zone under salt stress. The enzyme ACC-deaminase secreted by the microorganisms inhibit the ethylene induced negative changes in the root (Saleem et al., 2007; Nadeem et al., 2010a). Ethylene induction further inhibits auxin transcription factors, thus obstructing root growth. ACC deaminase secreted by the PGPR readily converts ACC (ethylene biosynthesis gene) to ammonia and α -ketobutyrate thus inducing root growth under stress conditions. The genus *Burkholderia* having the ability to synthesize the enzyme ACC deaminase promoted the growth of tomato seedlings (Onofre-Lemus et al., 2009) by limiting ethylene levels. Studies pertaining to salt stress, showed that inoculating PGPR synthesizing ACC-deaminase (*Klebsiella oxytoca* Rs-5) facilitated uptake of N,P,K and Ca (Yue et al., 2007). Comparative studies on two different PGPR strains (*Pseudomonas putida* and *Serratia proteamaculans*) capable of synthesizing ACC deaminase showed that *P. putida* greater mitigating ability than the other strain (Zahir et al., 2009).

22.2.9 Nutrient uptake and source-sink manipulation by PGPR

The role of rhizobacteria' in nutrient use efficiency is thrust upon, when it plays a role in enhancing the nutrient solubilization, mobilization of inorganic and organic nutrients, availability and then N_2 fixation and also to siderophores synthesis that makes available the Fe and Zn micronutrients for uptake by the plants. PGPR inoculation has proven effects in accomplishing synergistic uptake of other nutrients along with K^+ under salt stressed conditions, retaining water holding capacity (Mayak et al., 2004; Nadeem et al., 2006). PGPR application depicted an increase in P and N availability in maize plants inoculated with beneficial *Azotobacter* strains (Rojas-Tapias et al., 2012). *Azotobacter* PGPR strains also proved effective in mitigating salt stress in wheat plants without compromising biomass yield, nitrogen content and grain yield (Chaudhary et al., 2013). Co-inoculation of mung bean seeds with PGPR *Enterobacter* and *Pantoea* sp. aided in limited Na^+ uptake under salt stress conditions (Panwar et al., 2016). These species are effective in synthesizing IAA and siderophores with P-solubilizing ability. In general, *Pseudomonas* sp. is effective in solubilizing phosphorus from the soils. Lettuce plants inoculated with *Bacillus* sp. under stress conditions recorded increase in N, P, and K concentration by about 5%, 70%, and 50%, respectively, over the un-inoculated plants.

These PGPRs also modulate the source sink assimilation in crops under salt stress like alterations in the sugar metabolism, their transport and storage. PGPR helps in the maintenance of an active sink activity in the root. PGPR capable of synthesizing IAA, improved the root sink activity to sustain growth under salinity (Pérez-Alfocea et al., 2010). Synthesis of trehalose-6-phosphate by the PGPR at the rhizosphere acts as an osmotolerant in phaseolus bean under salt stress. The plants inoculated with *Rhizobium tropici* and *Paenibacillus polymyxa* showed significant improvement in nodule formation and N uptake (Figueiredo et al., 2008). Similarly, PGPR (*Bacillus aquimaris*) inoculation in wheat resulted in higher levels of total sugars, reducing sugars and biomass under salt stress conditions (Upadhyay and Singh, 2015). *Bacillus* sp. and *Arthrobacter oxidans* inoculation in Arabidopsis had significantly enhanced photosynthesis while inducing a systemic defense (Barriuso et al., 2008).

22.2.10 PGPR induced ROS (reactive oxygen species) defense

Accumulation of ROS is highly harmful and chronic in causing damage to the membrane structure almost impairing the photosystem. Hence, up regulation of antioxidant metabolism by PGPR is an essential trait to mitigate salt stress in plants. PGPRs also play a role in inducing antioxidant enzymes (Dodd and Pérez-Alfocea, 2012). Inoculation of tomato

and okra plants with *Enterobacter* EJ01 strain and UPMPR18 strain respectively, has revealed the production of enzymatic and non-enzymatic antioxidants under salt stress preventing ROS (Reactive Oxygen Species) damage (Kim et al., 2014; Habib et al., 2016). Increased antioxidant levels, suppressing the Na^+ uptake is reported to be the possible mechanism in mitigating salt stress of eggplant by *Pseudomonas* spp. (Fu et al., 2010). Protection under high saline conditions via activation of the antioxidant system in several crops including rice has been documented (Arora et al., 2012; Jha and Subramanian, 2014). Rhizobacterial inoculation (*B. pumilis* and *P. Pseudoalcaligenes*) activated a caspase like protease activity in regulating lipid peroxidation caused by ROS. PGPR inoculation also has the ability to activate non-enzymatic antioxidants like ascorbate and glutathione under saline stress in mungbean preventing the oxidative damage (Panwar et al., 2016).

22.2.11 Role of extracellular compounds for improving salt tolerance

The PGPRs synthesize, extracellular root secretions in the niche, which can regulate signaling events, improve defense against biotic and abiotic stresses. These secretory molecules include Exopolysaccharides, Lipochitopolysaccharides, polyamines, volatile organic compounds and bactericidal proteins that form a protective shield around the rhizoplane.

Exopolysaccharides improve water holding capacity, cation exchange capacity by forming a film at root zone to facilitate nodule formation, soil texture improvement and salt stress mitigation up to 400 mM NaCl (Upadhyay et al., 2011; Qurashi and Sabri, 2012; Sandhya and Ali, 2015, Yang et al., 2016). A study by Upadhyay et al. (2011) proved that EPS producing PGPR (*Paenibacillus* spp.) in wheat help in alleviating salt stress by reducing Na^+ uptake (sodium exclusion from roots), while Nadeem et al. (2010a,b) reported the counteraction of ethylene effects on pea while providing salt tolerance in 150 mM NaCl when inoculated with *Pseudomonas* sp.

Lipochitin oligosaccharide (LCOs) signal molecules called Nod factors have a chitin β -1 $\rightarrow$ 4-linked *N*-acetyl-D-glucosamine backbone (varying in length from three to six sugar units) and a fatty acyl chain on the C-2 position of the non-reducing sugar (Taiz and Zeiger, 2010). Host specific LCOs in response to PGPR inoculation favor nodule formation and improve N_2 fixation under excess salt concentrations in several crops including soybean (*B. japonicum*; Duzan et al., 2004), Phaseolus bean (*Rhizobium tropici*; Dardanelli et al., 2008). The small peptidal proteins called bacteriocins induce photosynthetic genes and stress tolerance related proteins in soybeans including glutathione-S-transferase that acts as an antioxidant (Subramanian et al., 2016a,b).

Production of secondary metabolites which include PAs (polyamines) by the colonizing PGPR (Zhou et al., 2016) are known to contribute to osmotic stress tolerance, improvement in ROS defense and even maintain plant water relations. The PA compounds accumulated in the cell that are determinants mediating these responses are spermine, spermidine and putrescine. Inoculation of *Bacillus subtilis* has been demonstrated to induce polyamine production under osmotic stress conditions (Xie et al., 2014). Apart from these, the plant volatile cues have been widely studied to regulate plant metabolism and defense, but its role in salt tolerance remains less explored. Some of the VOCs' that are responsible for salt stress tolerance under PGPR inoculation are cyclohexane, 2-(benzyloxy)ethanamine, benzene, methyl, decane, 1-(*N*-phenylcarbamyl)-2-morpholinocyclohexene, dodecane, benzene(1-methylnonadecyl), 1-chlorooctadecane, tetradecane, 2,6,10-trimethyl, dotriacontane and 11-decyldocosane (Kanchiswamy et al., 2015).

22.3 Conclusion

There is convincing evidence that PGPR responses are induced like increased tolerance to salt stress, nutrient use efficiency, plant growth and promotion. Apart from these, PGPRs also regulate and acclimatize plant under salt stress by influencing positive phytostimulatory hormones (IAA, CK, GA, strigolactones), production of extracellular molecules, enzymes such as phosphatase (to solubilize/chelate inorganic PO_4), siderophores, secondary messengers and genes. Also, the additive or synergistic effect of combined inoculation of PGPR strains has been more beneficial in ameliorating the stress effects on crop plants. Hence, there exists a need for bioprospecting, screening, compatibility studies, product formulation and overcoming the drawbacks like slow release and action suiting for both abiotic and biotic stress. This will be a viable alternative approach to chemical fertilizers as well as conventional biofertilizer application.

To address the salt stress mitigation with PGPRs, the following approach, methodology has to be adopted (i) isolation and characterization of the beneficial PGPR consortia or strain to the soil microbiome (ii) testing their growth and stability; synergism/antagonism, under wide range of stress environment (iii) to be able to promote plant/crop growth and yield in a largely significant manner (iv) most importantly the efficient strain/s to be suitable for low cost formulation, made available to the end users.

In toto, as per the reports of Nakkeeran et al. (2005), an PGPM should have higher competence at the rhizosphere/plane, have broad spectrum mode of action, be compatible with other rhizobia, enhance plant growth and development, be tolerant to temperature and other conditions in an ecofriendly and agriculturally sustainable manner. These studies hold great potential with combined or single inoculation to combat salt stress in an ecofriendly and sustainable way. Further, understanding the specific mechanisms employed by the PGPR to enhance tolerance will be favorable to employ them for other abiotic stress conditions.

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Nematophagous and entomopathogenic fungi: new insights into the beneficial fungus-plant interaction

Filippe Elias de Freitas Soares^{1,2}, Patrícia Silva Gôlo³ and Éverton Kort Kamp Fernandes¹

¹Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil, ²Departamento de Química, Universidade Federal de Lavras, Minas Gerais, Brazil, ³Departamento de Parasitologia Animal, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil.

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23.1 Introduction

Entomopathogenic and nematophagous fungi are microorganisms widely studied and applied on biological control of pests. The commercialization of these fungi is a reality, with many products available worldwide (Jaronski, 2014; Moosavi and Zare, 2012). Recent studies show that these fungi also have influence on rhizosphere. Microbial biological control coupled with plant growth provides alternatives to sustainable agricultural production (Palaniyandi et al., 2013). The endophytic potential of entomopathogenic and nematophagous fungi was extensively reviewed by Quesada-Moraga et al. (2014a); however, many new studies have been performed after that publication. Therefore, in this chapter, we focus on new researches, mostly conducted from 2014 to 2018 regarding the beneficial fungus-plant interaction.

23.2 Nematophagous fungi

Nematophagous fungi are cosmopolitan microorganisms, natural antagonists of nematodes that feed on several living stages of these animals (Nordbring-Hertz et al., 2006). Many of the nematophagous fungi are facultative parasites, and in the presence of their hosts they can change from a saprophytic to a parasitic stage and form infection structures, which are used to classify them according to their predation characteristics (Lopez-Llorca et al., 2006). These fungi can be classified into four groups: nematode-trapping/predators, opportunistic or ovicidal, endoparasites, and toxin-producing fungi. Currently, a new group of nematophagous fungi has been proposed: producers of special attack devices (Liu et al., 2009; Soares et al., 2018). Nematode-trapping fungi use various types of modified hyphae, e.g. adhesive networks, adhesive knobs or constricting rings, to capture nematodes by adhesion and mechanical function, and to digest and to eat them using enzymes (mainly proteases, chitinases and lipases). The ovicidal group also uses traps/enzymes to prey and to feed on eggs, cysts and nematode females. Endoparasitic fungi are a group of fungi that use

their conidia to infect nematodes. The fungi from this class have no saprophytic phase or only a limited saprophytic phase, and produce almost no mycelium in soil, hindering its cultivation and industrial use (Moosavi and Askary, 2015; Li et al., 2015). Toxin-producing fungi attack their prey by secreting diffusible toxins, immobilizing them before hyphae penetration through the cuticles (Lopez-Llorca et al., 2008). Special attacking devices are similar to a weapon, causing damage to the nematode cuticle and allowing fungal colonization and consumption (Soares et al., 2018).

Nematophagous fungi are used in biocontrol of phytonematodes, thus protecting plants from these parasites. Moreover, it is known that these fungi are found in the rhizosphere of crop plants and can colonize their roots (Bordallo et al., 2002; Lopez-Llorca et al., 2002). Plant-parasitic nematodes generally attack plant roots; therefore, the ability of nematophagous fungi to colonize roots is very attractive to explore (Bordallo et al., 2002).

23.3 Nematophagous fungi as endophytes

The literature reports nematophagous fungi act as endophytes and promote a number of beneficial effects on plants. Some species of nematophagous fungi have already been described with characteristics beneficial to plants and rhizosphere. Among them, the ovidical fungus *Pochonia chlamydosporialis* is the most prominent microorganism, with the highest number of published studies (Table 23.1).

Endophyte fungi are found in asymptomatic plant tissues without causing detectable damage to their host; endophytes may complete the whole life cycle or part of their life cycle while in the plant. The endophytic behavior was observed in all four classes of nematophagous fungi (Lopez-Llorca et al., 2006); however, studies focused on basidiomycetous endophytes are limited (Martin et al., 2015).

An interesting implication of the endophyte behavior of nematophagous fungi is their possible use for controlling the various life stages of nematodes by internal root colonization (Evans et al., 2017). Thus, a fungal application would have a double action: they would be beneficial to the host plant, and destructive against nematodes. Moreover, certain nematophagous fungi produce resistant structures (chlamydospores), and do not necessarily depend on a host for their reproduction (Monteiro et al., 2018).

23.4 Positive effects of the interaction of nematophagous fungi and plants

Several plants of commercially important crops have already been tested for the positive influence of nematophagous fungi. *Clonostachys rosea* improve the growth of carrot and wheat and decrease the populations of several plant-parasitic nematodes in naturally nematode-infested soil (Iqbal et al., 2017). Also, *Arthrobotrys oligospora*, *Dactylella oviparasitica*, *Clonostachys rosea*, *Stropharia rugosoannulata* and *Lecanicillium muscarium* have improved carrot growth parameters (Hussain et al., 2017). *Purpureocillium lilacinum* increased fresh and dry weight and number and weight of spikelets of oats plants (*Avena sativa*) (Hernández-Leal et al., 2016). Mwaheb et al. (2017) demonstrated that the combined application of *H. minnesotensis* and chitosan to soil increased the relative abundance of the core bacterial and fungal taxa in the assembled soybean rhizosphere microbial communities, which suppressed root infestation by the soybean cyst nematode. Moreover, *Pochonia chlamydosporia* was used successfully in experiments in tomato and maize crops (Luambano et al., 2015; Zavala-Gonzalez et al., 2015; Mostafanezhad et al., 2014). Larriba et al. (2015) demonstrated that *P. chlamydosporia* root colonization induces changes in the barley (*Hordeum vulgare*) transcriptome, which primarily include the up-regulation of genes involved in plant growth promotion.

In the context of the positive interaction of nematophagous fungi with plants, we can highlight the ability of some microorganisms to synthesize phytohormones that can improve the quality and productivity of cultivars with high economic importance. For example, *Lecanicillium psalliotae* exhibited direct plant growth by producing indole-3-acetic acid and ammonia (Kumar et al., 2018). *Paecilomyces lilacinus* produces indole acetic acid (IAA), which promotes tomato growth (Yu et al., 2015). The induction of barley growth by *P. chlamydosporia* association was concomitant with induction of the expression of phytohormone genes such as auxins, jasmonic acid and gibberilins (Larriba et al., 2015).

Besides acting as biocontrol agent and favoring the productivity of the host plants, certain nematophagous fungi act solubilizing and facilitating the availability of phosphate in the soil, thus benefiting the absorption of this mineral by the plants (Monteiro et al., 2018). *P. chlamydosporia* and *D. flagrans* increased the phosphorus acquisition by tomato plants, possibly by the solubilization of inorganic fractions of this mineral (Monteiro et al., 2018). Other two nematophagous fungi, *Geomyces pannorum* and *Paecilomyces carneus*, increased the available phosphorus in the soil by more than 30%, suggesting that these fungal species may have potential uses in agricultural soils with insoluble phosphorus (Lima-Rivera et al., 2016).

TABLE 23.1 Some recent reports of Nematophagous fungi promoting beneficial effects on plants.

Fungi	Effect	References
<i>Pochonia chlamydosporia</i> and <i>Duddingtonia flagrans</i>	Phosphorus uptake and plant growth	Monteiro et al. (2018)
<i>Pochonia chlamydosporia</i>	Accelerated flowering and improved yield	Zavala-Gonzalez et al. (2017)
<i>Pochonia chlamydosporia</i>	Up-regulation of genes involved in plant growth promotion	Larriba et al. (2015)
<i>Pochonia chlamydosporia</i>	Improved yield	Luambano et al. (2015)
<i>Pochonia chlamydosporia</i>	Increase in shoot weight	Podestá et al. (2016)
<i>Pochonia chlamydosporia</i>	Root growth and reduce flowering time	Zavala-Gonzalez et al. (2015)
<i>Pochonia chlamydosporia</i>	Plant growth	Sellitto et al. (2016)
<i>Pochonia chlamydosporia</i>	Plant growth	Dallemole-Giaretta et al. (2015)
<i>Pochonia chlamydosporia</i>	Chitosan combined with <i>P. chlamydosporia</i> promoted root colonization	Escudero et al. (2017)
<i>Pochonia chlamydosporia</i> , <i>Purpureocillium lilacinum</i> and <i>Trichoderma asperella</i>	Greater root development and increased mycorrhizal colonization	Farias et al. (2018)
<i>Lecanicillium psalliotae</i>	Plant growth	Kumar et al. (2018)
<i>Paecilomyces lilacinus</i>	Significant reduction in exchangeable NH_4^+ -N and NO_3^- -N contents	Yu et al. (2015)
<i>Purpureocillium lilacinum</i>	Plant growth	Lopez and Sword (2015)
<i>Purpureocillium lilacinum</i>	Increased fresh and dry weight and number and weight of spikelets	Hernández-Leal et al. (2016)
<i>Purpureocillium lilacinum</i>	Improved yield	Kepenekci et al. (2018)
<i>Paecilomyces lilacinus</i> and <i>Hirsutella rhossiliensis</i>	Plant growth	Abo-Korah (2017)
<i>Trichoderma asperellum</i> , <i>Pochonia chlamydosporia</i> , <i>Kuklopora kentinensis</i> , <i>Aspergillus allahabadii</i> and <i>Hirsutella minnesotensis</i>	Increased shoot and root fresh weights	Affokpon et al. (2018)
	Combined application of <i>H. minnesotensis</i> and chitosan led to shifts in the abundance of the core rhizobacterial and rhizofungal taxa	Mwaheeb et al. (2017)
<i>Geomyces pannorum</i> and <i>Paecilomyces carneus</i>	Increased the available phosphorus in the soil	Lima-Rivera et al. (2016)
<i>Arthrobotrys oligospora</i>	Increased fresh foliage weight	Mostafanezhad et al. (2014)
<i>Trichoderma harzianum</i> and <i>Trichoderma viride</i>	Plant growth	Al-Hazmi and TariqJaveed (2016)
<i>Trichoderma harzianum</i>	Plant growth	Rizvi et al. (2018)
<i>Arthrobotrys oligospora</i> , <i>Dactylella oviparasitica</i> , <i>Clonostachys rosea</i> , <i>Stropharia rugosoannulata</i> and <i>Lecanicillium muscarium</i>	Plant growth	Hussain et al. (2017)
<i>Clonostachys rosea</i>	Increased fresh shoot weight and shoot length	Iqbal et al. (2017)

Chitosan, a deacetylated derivative of chitin, is a linear polysaccharide of β -(1–4) linked 2-acetamide-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose (Nilsen-Nygaard et al., 2015). Chitosan improves the biocontrol efficacy of nematophagous fungi against plant parasitic nematodes. Also, it has shown synergic effect with these fungi on root colonization and microbial communities (Escudero et al., 2017; Mwaheb et al., 2017).

Some studies demonstrate the effects of plant *versus* nematophagous fungi interaction on the rhizosphere microbiota. Mwaheb et al. (2017) demonstrated the effect of chitosan and *Hirsutella minnesotensis* on the microbial populations of the soybean rhizosphere. Those authors have reported some interesting changes; for example, chitosan enhanced the abundance of Sphingomonadaceae, Streptomycetaceae, Intrasporengiaceae, Phyllobacteriaceae, Methylobacteriaceae and Pseudonocardiaceae. The combined application of *H. minnesotensis* and chitosan increased the abundance of the families Micrococcaceae, Sphingomonadaceae, Burkholderiaceae, Streptomycetaceae and Phyllobacteriaceae. In consideration to the fungal communities, chitosan-amended soil only resulted in increased abundance of Sordariomycetes. Both chitosan and *H. minnesotensis*, and *H. minnesotensis* individually increased abundance of Sordariomycetes; their combination, however, increased the abundance of Tremellomycetes, Dothideomycetes and Agaricomycetes, but not Sordariomycetes.

Arbuscular mycorrhizal colonization was studied in sugarcane, corn and soybean by applying some nematophagous and entomopathogenic fungi: *Beauveria bassiana*, *Metarhizium anisopliae*, *Pochonia chlamydosporia*, *Purpureocillium lilacinum* and *Trichoderma asperella* (Farias et al., 2018). In that study, the colonization rate of the inoculated treatment in comparison to the uninoculated treatment was 20% higher for soybean and 28% higher for sugarcane ($P < 0.05$). However, corn roots showed no differences in root length colonized by arbuscular mycorrhizal fungi between the two treatments. However, in another study, PCR-denaturing gradient gel electrophoresis (DGGE) analysis demonstrated no significant difference in soil fungal community composition of tomato rhizosphere associated with the application of *Paecilomyces lilacinus* (Yu et al., 2015).

P. chlamydosporia is undoubtedly the nematophagous fungus most studied regarding to its beneficial interaction with plants. In recent years, some studies have begun to elucidate the interaction of the fungus with various plants (Table 23.1). In addition to macroscopic effects on plants, some molecular interaction has also been studied. Gene expression changes in flowering time gene expression of *Arabidopsis thaliana* in response to the presence of *P. chlamydosporia* were evaluated by qPCR. The molecular results support that *P. chlamydosporia* promotes developmental progression, flowering and yield of *A. thaliana* (Zavala-Gonzalez et al., 2015). Escudero et al. (2014) performed a metabolomic approach to study the rhizodeposition of tomato, *P. chlamydosporia* and *Meloidogyne javanica* interaction. A large increase in an unassigned aromatic compound was detected by ^1H NMR in *P. chlamydosporia* colonized roots. The authors suggested that this data could be an evidence of induction of plant defenses by *P. chlamydosporia*. In another study (Larriba et al., 2015), the transcriptomic response of barley root to colonization by *P. chlamydosporia* was evaluated. In that study, of a total of 22,792 probesets, 1.2% showed an altered expression pattern during root endophytic colonization by *P. chlamydosporia*, with 259 (96.6%) probesets being up-regulated and 9 (3.4%) down-regulated. Genes implicated in the biosynthesis of plant hormones, such as auxin, ethylene and jasmonic acid were up-regulated.

23.5 Entomopathogenic fungi

The use of entomopathogenic fungi for insect control has been practiced and studied, with different approaches, for more than 100 years (Roberts and Leger, 2004). *Metarhizium* spp., and *Beauveria* spp. are among the fungal species most frequently used in the biological control of arthropod pests. Classical control of arthropod pests using entomopathogenic fungi is often performed by direct application of the fungal propagules to the plant field where the pests normally develop. The success of direct-product application in the field depends on subsequent development and multiplication of the fungus into the arthropod pest, thereby causing its death. Despite a series of abiotic stresses, such as low humidity, high incidence of sunlight, rain, and high temperatures, act as negative effectors on the action of the entomopathogenic fungi (Fernandes et al., 2015; Rangel et al., 2010; Rangel et al., 2005). The overwhelming majority of studies on entomopathogenic fungi and arthropod pests discusses the death of these arthropods due to direct fungal infection (Roberts and Leger, 2004; Faria and Wraight, 2007; Lacey et al., 2015; Mascarin et al., 2018). Interestingly, recent literature (starting almost three decades ago) has reported the suppression of insect damage in response to the colonization of entomopathogenic fungi in plants (Vega, 2018), what interestingly could reduce the abiotic conditions faced by these fungi, once the entomopathogens are inside the plant. Additional studies reported further roles for entomopathogenic endophytes, other than insect control, including positive effects on plant growth, plant disease antagonism, and rhizosphere colonization (Goettel et al., 2008; Pava-Ripoll et al., 2011; Wyrebeek et al., 2011; Behie et al., 2012;

Liao et al., 2017; McKinnon et al., 2018). The endophytic competence of entomopathogenic fungi may be a valuable (and insufficiently explored) characteristic of these fungi not only as entomopathogens, but also to improve plant development or benefit plant protection. As an example, a recent study of McKinnon et al. (2018) reported that maize (*Zea mays*) defense response changed under intense wounding of the foliage, resulting in the apparent recruitment of *Beauveria* sp. in the rhizosphere. This *Beauveria* sp. recruitment is suggested by the authors to be an indirect defensive strategy as a natural enemy of insect herbivores and/or the result of induced systemic susceptibility in maize, enabling plant colonization. Such knowledge is extremely relevant for both biological control of pests and the new-discovered plant protection roles by entomopathogenic fungi.

23.6 Entomopathogenic fungi as naturally endophytes or artificially inoculated into plants

Endophytes are microorganisms (bacteria, fungi, actinomycetes, or viruses) that can live within the plant tissues without causing disease (Wani et al., 2015). According to these authors, endophytes can be characterized into two general categories: systemic or true endophytes, and nonsystemic/transient endophytes; both categories are included in the widely accepted definition of endophytes, which is: organisms that live within the plant tissue at least for part of their life cycle without producing any apparent disease in plants under normal conditions. Fungal entomopathogens have been reported as naturally occurring endophytes (Table 2 in Vega Vega (2018)) or successfully artificially inoculate into plants using different inoculation methods (Table 1 in Vega (2018)).

Both transient plant colonization and vertical transmission were reported for entomopathogenic fungi (Quesada-Moraga et al., 2014b; Russo et al., 2015; Lefort et al., 2016; Renuka et al., 2016; Garrido-Jurado et al., 2017; Rondot and Reineke, 2018; Sánchez-Rodríguez et al., 2018). Vertical movement of entomopathogenic fungi were also observed by Quesada-Moraga et al., 2014b that used opium poppy plants and *B. bassiana* to first demonstrate the vertical plant transmission of this fungus. According to this publication, at the end of notching, *B. bassiana* was detected mainly at the basal part of the plant, whereas at capsule formation it was detected inside the leaves at the plant tip, indicating that the fungus had adapted its growth strategy to reach the reproductive tissues, ensuring transmission to progeny from endophytically colonized maternal plants.

Despite the frequent and relatively new literature on entomopathogenic fungi as endophytes in different plant species (Lefort et al., 2016; Renuka et al., 2016; Garrido-Jurado et al., 2017; Rondot and Reineke, 2018; Sánchez-Rodríguez et al., 2018; Shrivastava et al., 2015; Vidal and Jaber, 2015; Gathage et al., 2016; Gautam et al., 2016; Greenfield et al., 2016; Jaber and Enkerli, 2016; Klieber and Reineke, 2016; Mutune et al., 2016; Ramírez-Rodríguez and Sánchez-Peña, 2016a; Ramírez-Rodríguez and Sánchez-Peña, 2016b; Resquín-Romero et al., 2016; Jaber and Enkerli, 2017; Jaber and Araj, 2018; Parsa et al., 2018), a recent review on *B. bassiana* as an endophyte from McKinnon et al. (2017) emphasized the need for stringent protocols for future evaluation of experimental *B. bassiana* endophytic colonization, and recommended combining several methods of fungal detection to acquire a clear perspective of the plant-host interaction. They also suggested (I) testing the effectiveness of surface sterilization techniques for each unique plant species; (II) always use experimental controls to verify surface sterilization efficacy; and (III) exclude DNA from surface inoculum when using molecular detection (PCR) protocols.

23.7 Endophytic fungal entomopathogens and plant growth

Crop production gains originate mostly from improved cropping techniques, fertilization and irrigation. However, the production expansion can be associated with relevant negative environmental effects. Accordingly, there is an increasing demand for more sustainable solutions to raise yields. As stated by Tall and Meyling (2018), plant productivity could be enhanced by exploring the potential of beneficial plant-microbe interactions. Most studies that approached plant responses to endophytic entomopathogenic fungi reported a beneficial interaction for the plant (see Vega (2018), supplementary Table 1). Increased plant growth caused by entomopathogens is suggested to be a response to suppression of several biotic and abiotic stresses, including plant diseases (Sasan and Bidochka, 2013; Jaber and Salem, 2014; Jaber and Ownley, 2018). The latest study also discussed mycoparasitism, competition for ecological niches, competition for nutrition, and production of secondary metabolites as possible means involved in phytopathogens suppression.

Experiments on nitrogen translocation showed that several *Metarhizium* species and *B. bassiana* have the capacity to transfer nitrogen from dead insects to plants, although to various degrees (Behie et al., 2012; Behie and Bidochka, 2014). According to the latest study, endophytic association by these fungi increased overall plant productivity. One of the mechanisms responsible for the *Metarhizium* mediated plant growth boost was unveiled by Liao et al. (2017) that

reported *M. robertsii* promotes lateral root growth and root hair development of *Arabidopsis* seedlings in part by an auxin [indole-3-acetic acid (IAA)]-dependent mechanism.

It is also known that the plant growth promotion by *B. bassiana* is context dependent and may rely on nutrient availability (Tall and Meyling, 2018). These authors reported that maize seeds treated with *B. bassiana* GHA isolate increased the growth of experimental plants at high nutrient conditions, whereas under low nutrient availability *B. bassiana* did not affect maize growth. The above cited literature showed that the potential use of entomopathogenic fungi in agriculture may be underexploited; however, the mode of action of entomopathogenic fungi as plant growth promoters shall be fully elucidated before these fungi being used in the field.

23.8 Effects of endophytic and rhizosphere competent entomopathogenic fungi for insect pest control

The endophytic fungal entomopathogen potential for biological control of insect pests have been reported since 1990 (Vega, 2018; Vakili, 1990), despite most publications on the use of entomopathogenic fungi against arthropods discuss the insect death due to direct infection by fungal propagules. In a pioneering study, Bing and Lewis (Bing and Lewis, 1991) observed that tunneling in corn plants by *Ostrinia nubilalis* larvae, the European corn borer, was reduced when plants were endophytically colonized by *B. bassiana*. Since then, different entomopathogenic fungal species have been reported to reduce insect damage in several crops, highlighting the most recent (Lopez and Sword, 2015; Mutune et al., 2016; Akutse et al., 2013; Castillo-Lopez et al., 2014; Mantzoukas et al., 2015; Peña-Peña et al., 2015). Possible mechanisms that do not depend on direct insect infection by the fungal entomopathogen have been suggested to be involved in insect suppression by entomopathogenic-fungus-colonized plants, but yet unconfirmed (Vega, 2018; Jaber and Ownley, 2018). Among these mechanisms are: (i) plant resistance induced by the entomopathogenic fungus (Lopez and Sword, 2015; Castillo-Lopez et al., 2014), and (ii) production of secondary metabolites *in planta* (Garrido-Jurado et al., 2017; Mantzoukas et al., 2015; Golo et al., 2014) that could cause feeding deterrence and antibiosis to herbivorous insect. Despite this, to prove the real effect of *in planta*-entomopathogens for insect control, it would be necessary to access the effects of entomopathogenic fungi in endophyte-free plants (what is challenging) (Vega, 2018).

The persistence of applied entomopathogenic fungi in the environment could be supported not only by the fungus-plant endophytic interaction, but also by the fungal rhizosphere competence. The ability of a microorganism to colonize and establish in the rhizosphere of developing roots has been defined as rhizosphere competence (St Leger, 2008). The soil persistence of a rhizosphere competent fungus is particularly convenient when the arthropod pest target is not a chewing insect but lives on the ground. Kabaluk et al. (2017) associated the number of *Metarhizium brunneum*-infected wireworms to the abundance of this fungus in the collected soil samples. As a result, they found that the largest number of infected insects came from sites where *M. brunneum* was more prevalent. For insect-pests present in the soil, the appropriate saprophytic growth of *Metarhizium* spp. in the rhizosphere preserve the fungus in the environment until it finds an arthropod host.

Accordingly, it is possible to establish an ecological relationship between entomopathogenic fungi and plants, when they are present in the same environment. This endophytic capacity may imply an alternative way of fungi to control arthropod-pests other than the application of fungal-propagules to contact the arthropods. In addition, inside the plants, these fungi are protected from negative abiotic factors such as solar radiation, high temperatures and low humidity, characterized as important obstacles for the application of entomopathogenic fungal propagules in the field.

23.9 Evolutionary transition and molecular aspects of entomopathogenic endophytes

Biological control of arthropod pests, especially insects, has its basis in the earliest records of diseases that affected insects. According to Steinhaus (1975), the records of diseases in bees date from several centuries before Christ (approximately 700 BCE). Problems with the death of silkworms in sericulture have also been a subject of important studies in Europe of past centuries. Even before knowing the microorganisms, men aimed to fight “green and white muscardines” (in reference to the colors of the fungi that produced the insect disease, now known as *Metarhizium* sp. and *Beauveria* sp.). Accordingly, these microorganisms were able to attack and kill insects that men started to use these entomopathogens to control pests that destroyed their food crops, and thus the biological control with the use of entomopathogenic fungi (Steinhaus, 1975).

Despite the role of these fungi on “killing insects”, advanced methods applied to phylogenetic analyzes suggest that *Metarhizium* evolved from fungi that were associated to plants, and that the insect pathogenicity was a latest association (Gao et al., 2011; Barelli et al., 2016). It is also suggested that association with plants plays a major role in the divergence of *Metarhizium* genus rather than the insect-fungus relationship (Wyrebek and Bidochka, 2013). In fact, *Metarhizium* is now considered a fungus with a multifunctional lifestyle acting as an entomopathogen, saprobe, endophyte and plant growth promoter (Lacey et al., 2015; Jaber and Enkerli, 2016; Camargo et al; Lovett and St Leger, 2015).

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Secondary metabolites and lytic tool box of trichoderma and their role in plant health

Nidhi Kumari¹ and S. Srividhya²

¹Division of Crop Protection, ICAR-Central Institute for Subtropical Horticulture, Lucknow, India, ²Division of Field Crop improvement and Protection, ICAR- Central Island Agricultural Research Institute, Port Blair, India

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24.1 Introduction

History shows that crop diseases had resulted in starvation, hunger and mass deaths. At present crop diseases result up to 40% losses to the global agricultural productivity annually (Savary et al., 2012). Since 1960, with introduction of organophosphates, synthetic insecticides have contributed greatly in plant diseases management and therefore multiplying the agricultural outputs to many times (Aktar et al., 2009). But their unbridled use has resulted in huge financial strain on farmers, some major environmental issues, debit to human and other live forms (Aktar et al., 2009). Therefore, biological control having reduced environmental impact with increasing public demand and acceptance, serve as an alternative to chemical pesticides (Reino et al., 2008). An outstanding ascomycetous fungi genus, *Trichoderma* (Teleomorph *Hypocrea*) (Division - Ascomycota, Subdivision - Pezizomycotina, Class - Sordariomycetes, Order - Hypocreales, Family – *Hypocreaceae*), first reported and described by Persoon in 1794, is one of the most widely researched genera of filamentous fungi (Lee et al., 2016), holds the topmost rank in the list of fungal biocontrol agents (BCA) (Whipps and Lumsden, 2001). In 1934, Weindling introduced about the biological activity of *Trichoderma* against plant pathogens by demonstrating the biocontrol activity of *T. lignorum* against *Rhizoctonia solani* and other soil fungi. Since then *Trichoderma* has fascinated scientists from all across the globe because of its biocontrol potential against many phytopathogens. The major landmark discoveries in *Trichoderma* research have been presented in Table 24.1. *Trichoderma*, a cosmopolitan fungus, live in soil and saprophytically grow on different substrates like wood, bark etc. are highly adaptive to different ecological conditions (Zeilinger et al., 2016). *Trichoderma* spp. are common soil and root inhabitant (Contreras-Cornejo et al., 2016) active against different soil borne pathogens viz. *R. solani*, *Sclerotium rolfii*, and *Pythium* spp. *Trichoderma* genus currently accommodates more than 260 species (Plessis et al., 2018). The mechanism of *Trichoderma*'s biocontrol activity includes mycoparasitism (direct contention with phytopathogens through secretion of cell wall degrading enzymes followed by penetration of the target organism) (Woo and Lorito, 2007), antibiosis (secretion of antimicrobial secondary metabolites e.g. gliotoxin, gliovirin, viridin, and trichoviridin) (Sivasithamparam and Ghisalberti, 1998), direct competition for nutrients and space (Benitez et al., 2004;

TABLE 24.1 The major landmark discoveries in *Trichoderma* research.

S. No.	Year	Discovery	Reference
1.	1932	First evidence of mycoparasitic nature and biocontrol potential of <i>Trichoderma</i> spp.	Weindling and Emerson (1936)
2.	1934	Discovery of first antimicrobial compound Gliotoxin from <i>Trichoderma</i> spp.	Weindling (1934)
3.	1972	Demonstration of biological suppression of <i>Sclerotium rolfsii</i> by <i>T. harzianum</i> in field	Wells et al. (1972)
4.	1979	Isolation of cellulase hyperproducing mutant RutC30 of <i>T. reesei</i>	Montenecourt & Eveleigh (1979)
5.	1983	Cloning of first cellulase of <i>T. reesei</i>	Shoemaker et al. (1983)
6.	1986	Demonstration of plant growth promotion by <i>Trichoderma</i>	Chang et al. (1986)
7.	1987	Successful transformation of <i>T. reesei</i>	Penttilä et al. (1987)
8.	1989	Heterologous protein expression in <i>T. reesei</i>	Harkki et al. (1989)
9.	1989	Registration of first commercial <i>Trichoderma</i> formulation for biocontrol under the name BinabT	-
10.	1992	Direct evidence for the role of lectines in mycoparasitism	Inbar & Chet (1992)
11.	1993	Cloning of first <i>prb1</i> gene (mycoparasitism related gene) and its induction by cell walls	Geremia et al. (1993)
12.	1994	Development of first technique capable of distinguishing a commercially used biocontrol strain, <i>T. harzianum</i> T-39 against <i>B. cinerea</i> , from other <i>Trichoderma</i> strains	Zimand et al. (1994)
13.	1997	Discovery of <i>Trichoderma</i> 's ability to induce systemic resistance in plants	Bigirimana et al. (1997)
14.	1999	Demonstration of internal coiling of plant roots by <i>Trichoderma</i> spp. and induction of systemic resistance in cucumber plants by <i>T. harzianum</i>	Yedidia et al. (1999)
15.	2002	Establishment of the role of G-proteins; Demonstration of involvement of signaling pathways in biocontrol and conidiation	Rocha-Ramirez et al. (2002)
16.	2002	Identification of peptaibols from <i>T. virens</i> and cloning of a peptaibol synthetase	Wiest et al. (2002)
17.	2003	Demonstration of negative regulation of conidiation in <i>T. virens</i> by MAPK	Mendoza-Mendoza et al. (2003), Mukherjee et al. (2003)
18.	2004	Discovery of photoreceptors (Brl1 and Brl2) in <i>T. atroviridae</i>	Casas-Flores et al. (2004)
19.	2005	Role of <i>Trichoderma</i> MAPK in ISR	Viterbo et al. (2005)
20.	2006	Discovery of protein elicitors (Sm1/Epl1) that induce plant defense response	Djonovic et al. (2006), Seidl et al. (2006),
21.	2006	Report of Xyr1, regulator of both hydrolytic enzyme system and D-xylose metabolism in <i>H. jecorina</i>	Stricker et al. (2006)
22.	2008	First <i>Trichoderma</i> (<i>T. reesei</i>) genome sequenced	Martinez et al. (2008)
23.	2009	Identification of <i>Thctf1</i> , transcription factor of <i>T. harzianum</i> involved in 6-pentyl-2H-pyran-2-one production and antifungal activity	Rubio et al. (2009)
24.	2009	First report of mating type locus MAT1–2 <i>T. reesei</i> and identification of its respective mating type counterpart, MAT1–1, thus proving its heterothallic nature	Seidl et al. (2009)
25.	2009	Identification of plant derived sucrose as a key element in symbiotic association of <i>T. virens</i> and maize plants	Vargas et al. (2009, 2011)
26.	2009	Demonstration of biomass production and lateral root growth in Arabidopsis by <i>T. virens</i> through an auxin-dependent mechanism.	Contreras-Cornejo et al. (2009)
27.	2010	Identification of VELVET protein, Vel1 regulator of morphogenesis and biocontrol properties in <i>T. virens</i>	Mukherjee & Kenerley (2010)

(Continued)

TABLE 24.1 (Continued)

S. No.	Year	Discovery	Reference
28.	2010	Description of a novel class of peptide pheromone precursors <i>hpp1</i> in <i>Trichoderma</i> spp.	Schmoll et al. (2010)
29.	2011	Identification of loci and functional characterization of trichothecene biosynthesis genes in <i>Trichoderma</i>	Cardoza et al. (2011)
30.	2012	Full genome sequencing of <i>T. harzianum</i> , <i>T. asperellum</i> , <i>T. longibrachiatum</i> , and <i>T. citrinoviride</i> using Next Generation Sequencing	-
31.	2012	Development of a construction kit for gene knock out in <i>T. reesei</i>	Schuster et al. (2012)
32.	2015	Draft genome sequence of <i>T. atroviride</i>	Shi-Kunne et al. (2015)
30.	2015	Introduction of a tool based on the use of simple sequence repeats (SSRs) using Touchdown PCR to track and quantify the <i>T. virens</i>	Geistlinger et al. (2015)
31.	2017	Development of multiplex PCR assay for monitoring the population levels of <i>T. atroviride</i> and <i>T. harzianum</i>	Oskiera et al. (2017)
32.	2017	Draft genome sequence of <i>T. harzianum</i>	Compant et al. (2017)

Verma et al., 2007), modification of rhizosphere thereby inhibiting the growth of phytopathogenic fungi (Harman et al., 2004). *Trichoderma* infestation results in improved plant growth by promoting nutrient uptake (Yedidia et al., 2003) as well as plant defensive mechanisms. *Trichoderma* spp. are abundant producer of antimicrobial secondary metabolites like non-ribosomal peptides (NRPs), peptaibols, polyketides, pyrones, siderophores, volatile and non-volatile terpenes (Velazquez-Robledo et al., 2011; Muller et al., 2013) which are not crucial for their primary metabolic processes. Secondary metabolites are low molecular weight natural compounds produced by an individual genus, species or strains of microorganisms and plants biosynthesized from primary metabolites in any specialized pathway (Vinale et al., 2014). The *Trichoderma*'s ability to produce a wide array of antimicrobial secondary metabolites with pharmaceutical and biotechnological importance play a crucial role in imparting the antagonistic biocontrol activity to the genus (Ajith and Lakshmidhevi, 2010). After the discovery of gliotoxin in 1936 by Weindling and Emerson, a plenitude of secondary metabolites produced by this genus were reported by different workers (Hermosa et al., 2014) of which structure of 120 secondary metabolites have been reported (Sivasithamparam and Ghisalberti, 1998; Reino et al., 2008). Therefore, in this chapter we shall discuss the wide array of secondary metabolites produced by *Trichoderma* spp., emphasizing their properties, structures, pharmaceutical and biotechnological roles and their application in plant disease management.

24.2 Regulation of secondary metabolism in *Trichoderma* Spp

The regulation of secondary metabolites synthesis is a complex mechanism and is often associated with sporulation in fungi. Generally, gene clusters bearing one or several central genes that encode large multidomain and multimodular core enzymes viz., polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs) or terpene synthases/cyclases, accessory enzymes like cytochrome P450s, oxidoreductases, methyl transferases, etc. (Hertweck, 2009; Strieker et al., 2010) regulate the synthesis of secondary metabolism in fungi unlike the genes involved in the primary metabolite synthesis which are scattered throughout the fungal genome. A typical gene cluster is minimally constituted of backbone/core enzyme (synthase and/or synthetase) that forms the carbon backbone from primary metabolites, thus define the chemical class of secondary metabolite produced and tailoring enzymes that further modifies the products of core enzymes. In addition to these two components large gene clusters also contain transcription factors which are the gene cluster specific or broad domain specific regulatory protein. Many genes which remain silent under standard laboratory conditions are referred to as "cryptic" or "orphan" genes. Scientists were successful to discover novel compounds by activating these silent genes either through mimicking microbial competition, or editing the master regulator genes, or constitutive overexpression of cluster-specific transcription factor, where present (Bergmann et al., 2007; Brakhage et al., 2008). Like other fungi, the expression of these genes in *Trichoderma* spp. are being influenced by presence of other microorganisms, soil environments, root exudates, pH etc (Atanasova et al., 2013a,b; Zeilinger et al., 2016). The transcription factor is inherent to the gene clusters and thus any change results in either no production or overproduction of secondary metabolites (Brakhage, 2013). The heterotrimeric velvet complex VelB/VeA/LaeA, a universal

regulator of secondary metabolism in fungi (Bayram et al., 2008) which in addition to its role in fungal development and secondary metabolism of *Aspergillus*, also coordinates light signal. In *T. virens*, the gliotoxin biosynthesis and gene clusters are under the regulation of veA ortholog vel. Determination of full *Trichoderma* genome sequence (*T. reesei*, *T. virens*, *T. longibrachiatum*, *T. atroviride*, *T. asperellum*, *T. hamatum*, *T. harzianum*, *T. citrinoviride*, *T. brevicompactum*, *T. gamsii*, *T. atrobrunneum*, *T. arundinaceum*, *T. koningiopsis*, *T. guizhouense*, *T. koningii*, *T. pleuroti*, *T. parareesei*) followed by their comparative analysis yielded valuable information regarding the genes involved in secondary metabolism of genus *Trichoderma*. Bansal and Mukherjee (2016) identified novel gene clusters involved in *Trichoderma*'s secondary metabolism by surveying three *Trichoderma* genomes viz. *T. reesei*, *T. atroviride* and *T. virens*. The genome of *T. virens*, *T. atroviride* and *T. reesei* contain 440, 349 and 262 genes, respectively involved either in biosynthesis, transport or catabolism of secondary metabolites (Kubicek et al., 2011). Non-ribosomal peptides synthetases (NRPS), polyketides synthases (PKS) and terpene cyclase were identified as core genes involved in *Trichoderma*'s secondary metabolism (Zeilinger et al., 2016). Wiest et al. (2002) identified 62.8 kb *tex1* gene coding for peptaibol synthetase, an enzyme involved in biosynthesis of the 18-residue peptaibol. Rubio et al. (2009) clone and characterized *Thctf1* gene from *T. harzianum* involved in 6-pentyl-2H-pyran-2-one production.

24.3 Different secondary metabolites produced by *Trichoderma*

Guerrero et al. (2008) implicated the role of cell wall degrading enzymes (CWDE) and secondary metabolites produced by *Trichoderma* spp. in its antagonistic properties. About 373 structurally diverse metabolites have been identified according to the Antibase database (Laatsch, 2012), which perform specific function at very low concentrations. However, of many, the specific activity of different metabolites is still unknown. But these metabolites are probably involved in chemical communication among soil inhabitants as mediators (Sivasithamparam and Ghisalberti, 1998; Reino et al., 2008). *Trichoderma* spp. produce numerous secondary metabolites such as anthraquinones, daucanes, pyrones, koniginins, viridins, viridifungins, N-heterocyclic compounds, trichodenones and cyclopentenone derivatives, ergosterol derivatives etc (Vinale et al., 2006; Xiao-Yan et al., 2006; Vinale et al., 2009). Reino et al. (2008), Sivasithamparam and Ghisalberti (1998) and Szekeres et al. (2005) based on the structure, suggested two classes of secondary metabolites. In one class, the volatile metabolites with low molecular weight, which include polyketides such as pyrones, simple aromatic compounds, isocyanate metabolites butenolides, and volatile terpenes are categorized while in other polar metabolites like diketopiperazin and peptaibols with high-molecular weight are included. Different classes of metabolites along with their brief history, origin, characteristics and examples are discussed in brief in the following sections.

24.3.1 Non-ribosomal peptides

24.3.1.1 Peptaibols

Peptaibols are non-ribosomal antibiotic peptides characteristically have higher content of 2-amino-isobutyric acid (Aib) as well as isovaline (Iva), an alkyl N terminus (usually acetyl) and a hydroxy-amino acid at the C terminus. The length remains short, typically between 15 and 20 residues although shorter ones are also known. The name peptaibol has been constructed from its structural components viz., peptide, Aib, and amino alcohol (Leitgeb et al., 2007). *Trichoderma* and another genus *Emericella* are main source of peptaibols. Their antimicrobial properties are due to the formation of pores in lipid membranes which leads to loss in osmotic balance and ultimately cell death (Bortolus et al., 2013). Non-ribosomal peptide synthetases (NRPS) a modular enzyme whose each module can be further subdivided into distinguishable domains (adenylation-A, thiolation-T and condensation-C), responsible for the recognition, activation and modification of amino acids that comprise the final peptide synthesize peptaibols (Marahiel, 1997; Cardoza et al., 2005). Suzukacillin and alamethicin were the first peptaibols isolated from *T. viride* strains 63C-I and NRRL 3199, respectively in 1966 (Ooka et al., 1966). The primary structure of alamethicin that took 10 years of research is given in Fig. 24.1A. Till date there are total 317 sequences of peptaibols submitted in peptaibol database (<http://peptaibol.cryst.bbk.ac.uk/home.shtml>). Peptaibols are divided into three subclasses (1) long sequence peptaibols containing 18–20 amino acid residues, for instance alamethicins (2) short-sequence peptaibols with 11–16 residues, for instance harzianins or zervamicins, and (3) ipopeptaibols with 7 or 11 residues. *T. koningiopsis* is known to produce trikoningin KA V, trikoningin KB I and KB II (Fig. 24.1B and C). Weist et al. (2002) characterized a 62.8-kb continuous open reading frame (ORF) which encodes for peptaibol synthetase whose disruption through mutation eliminates all peptaibol isoforms production from *T. virens*. Marik et al. (2018) investigated the peptaibiomes of *T. gamsii* and

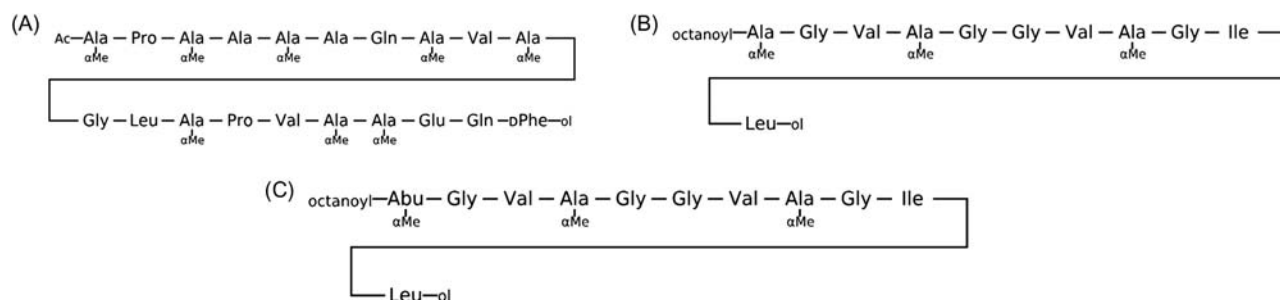


FIGURE 24.1 Biologic depiction of Alamethicin from *Trichoderma Viride* (A), Trikonigin KB I (B) and KB II (C) from *T. koningiopsis*.

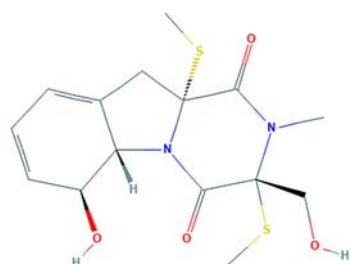


FIGURE 24.2 2D structure of Gliotoxin isolated from *Trichoderma virens*.

T. koningiopsis using HPLC-ESI-MS measurements, identified and characterized antimicrobial activity of 30 peptaibol against a wide range of microorganisms.

24.3.1.2 Epipolythiodioxopiperazines

Epipolythiodioxopiperazines (ETPs) are derived from cyclic peptides having an internal disulfide bridge which imparts toxicity to these molecules by generating reactive oxygen species through redox reaction (Mullbacher et al., 1986; Gardiner et al., 2005). On the basis of antibiotic production, the *Trichoderma* strains can be divided into “Q” (which produces diketopiperazine antibiotic gliotoxin) and “P” strains (that produce closely related compound, gliovirin which has inhibitory effect against Oomycetes only) (Howell and Stipanovic, 1983; Howell et al., 1993). Gliotoxin was first discovered by Weindling in 1934 from *T. virens*. Gliotoxin (Fig. 24.2) possess a wide spectrum of inhibitory effects on fungi, bacteria and viruses by preventing the fungal spore germination, bacterial multiplication and viral multiplication in tissue culture, respectively (Brian and Hemming, 1945; Rightsel et al., 1964). A NRPs, *GliP* which catalyzes dipeptide formation (a fusion of L-phenylalanine and L-serine) followed by cyclization to yield the diketopiperazine (DKP) scaffold, is core enzyme involved in the biosynthesis of gliotoxin (Gardiner & Howlett, 2005; Nierman et al., 2005). Vargas et al. (2014) observed that mutants with disrupted *GliP* locus which were generated using gene disruption strategy were incapable of producing gliotoxin. In addition to *GliP* locus, 12 other genes regulated by velvet complex proteins are co-regulated during gliotoxin biosynthesis (Schrettl et al., 2010; Dhingra et al., 2012). The antifungal activity of gliotoxin against *Fusarium*, *Alternaria*, *P. ultimum* and *R. solani* has been reported (Lewis & Lumsden, 2001; Dubey et al., 2011 Puluputuri et al., 2014;).

24.3.1.3 Siderophores

Siderophores are the low molecular weight ferric ion (Fe^{3+}) chelating metabolites that are produced by many microorganisms when grown under iron stress (Anke et al., 1991). Siderophores solubilize iron and make it available to plants thereby depriving the pathogenic microorganism from this essential nutrient (Leong, 1986). On an average, *Trichoderma* spp. produce 12–14 siderophores (Lehner et al., 2013). A general biosynthetic pathway for fungal siderophores was proposed by Plattner and Diekmann (1994). L-ornithine- N^5 -hydroxylase catalyze the N-hydroxylation of L-ornithine to N^5 -hydroxy-L-ornithine followed by acylation of N^5 -hydroxy-L-ornithine to form N^5 -acyl- N^5 -hydroxy-L-ornithine which is the basic structural unit of most fungal siderophores then condensation of several of the N^5 -acyl- N^5 -hydroxy-L-ornithine unit with the aid of NRPSs that covalently link these units via ester or peptide bonds to yield linear or cyclic peptides which subsequently can be further modified to synthesize a multitude of distinct siderophores (Renshaw et al., 2002; Lehner et al., 2013). Majority of the siderophores contain either catecholate or hydroxamate functional groups though most of fungi produce hydroxamate-type siderophores derived from L-ornithine (Plattner and Diekmann, 1994) with

Zygomycetes as an exception (Renshaw et al., 2002). Lehner et al. (2013) suggested that the diversity of siderophores might have resulted from further modifications in NRPS but not due to the diverse NRPS encoding genes. Coprogen B, ferricrocin and coprogen are secreted by all the six *Trichoderma* spp. (*T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride*, *T. longibrachiatum* and *T. pseudokoningii*) under iron deficient conditions (Anke et al., 1991). Vinale et al. (2009) purified harzianic acid, a nitrogen heterocyclic compound from *T. harzianum* and demonstrated its plant growth promoting ability and Fe^{3+} chelating ability (Vinale et al., 2013).

24.3.2 Polyketides

Polyketides are the polymers synthesized by polyketide synthase (PKS) enzyme, a multifunctional enzyme that is at least composed of ketosynthase, acyl-transferase and acyl carrier protein domains that catalyzes the fusion of coenzyme A (CoA) linked acyl monomers (Atanasova et al., 2013a,b). Kubicek et al. (2011) and Martinez et al. (2008) analyzed *Trichoderma* genome sequences and reported *T. virens* and *T. atroviride* encode 18 while *T. reesei* encode 11 predicted PKS. A phylogenomic analysis of PKS encoding enzymes by performing comparative genome analysis of *T. reesei*, *T. atroviride* and *T. virens* revealed that majority of PKS genes belong to orthologous group and fall on lovastatin/citrinin or fumonisins clades (Baker et al., 2012). Among different secondary metabolites, PKS are less studied. In *Trichoderma*, a large array of structurally diverse secondary metabolites has been reported.

24.3.2.1 Pyrones

The pyrone 6-pentyl-2H-pyran-2-one (6-PP) (Fig. 24.3) originally isolated from *T. viride* is a low-molecular weight metabolite. This metabolite is responsible for the characteristic “coconut aroma” (Collins & Halim, 1972), though also has been reported to have plant growth-boosting as well as anti-fungal properties (Vinale et al., 2008). 6-PP also have inhibitory effect on *Aspergillus flavus*, a potent producer of aflatoxin, thus may have practical application in stored food grains by eliminating the production of aflatoxin (Cutler et al., 1986). Scarselletti and Faul (1994) investigated the in vitro efficiency of 6-PP from *T. harzianum* IMI 288012 in the inhibition of two soil fungi viz., *F. oxysporum* f. sp. *lycopersici* and *R. solani*. They found 31.7% and 69.6% reduction in the growth of *F. oxysporum* f. sp. *lycopersici* and *R. solani*, respectively by adding 0.3 mg/ml 6-PP to agar medium. The effect of 6-PP on germination of *Fusarium* spores was also studied and it was observed that in aqueous solutions containing 0.45 mg/mL of 6-PP or greater, the spores of fungus did not germinate at all. Therefore, Scarselletti and Faul (1994) observed a strong positive correlation between the pyrone production by *T. harzianum* and its in vitro antagonistic ability. Apart from these fungi, pyrones from *Trichoderma* is inhibitory to *Botrytis*, *Sclerotinia*, *Pyrenochaeta*, *Athelia rolfsii* and *Staphylococcus aureus* (Claydon et al., 1987; Poole et al., 1998; Dodd et al., 2000; Evidente et al., 2003). Also the 6-PP from *T. harzianum* has fungistatic effect against *F. moniliforme* Sheld, one of the most important pathogens of maize (Hasan et al., 2007). The yield of 6-PP is higher in case of solid state fermentation (SSF) than submerged fermentation (Sarhy-Bagnon et al., 2000). Furthermore, the cultural parameters have effect on the production of pyrones in solid state fermentation. Ramos et al. (2008) studied several cultural parameters that enhance the pyrone production by *T. harzianum* 4040 in solid-state fermentation.

24.3.2.2 Koninginins

Liu et al. (2016) isolated and elucidated the structure of eight novel polyketides, Koningiopisins A-H and four previously identified polyketides viz., trichodermaketone C (Fig. 24.4A) (Song et al., 2010), koningin F (Fig. 24.4B) (Liu and Wang, 2001), koningin A (Fig. 24.4C) (Almassi et al., 1991) and koningin B (Fig. 24.4D) (Liu and Wang, 2001) from *T. koningiopsis*. Two new Koninginins R-S were isolated from *T. koningiopsis* and also shown to have anti-fungal activities against phytopathogens, *F. flocciferum* and *F. oxysporum* by Hu et al. (2017). Shi et al. (2017) isolated and characterized seven polyketides viz., 1, 6-di-epi-koninginin, ent-koninginin A, 10-deacetylkoningiopisin D, 15-hydroxykoninginin, trichoketide A, koningin T, and koningin L from *T. koningiopsis* strain QA-3.

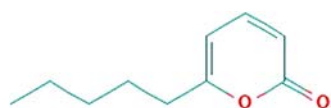


FIGURE 24.3 2D structure of 6-pentyl-2H-Pyran-2-one (6-PP).

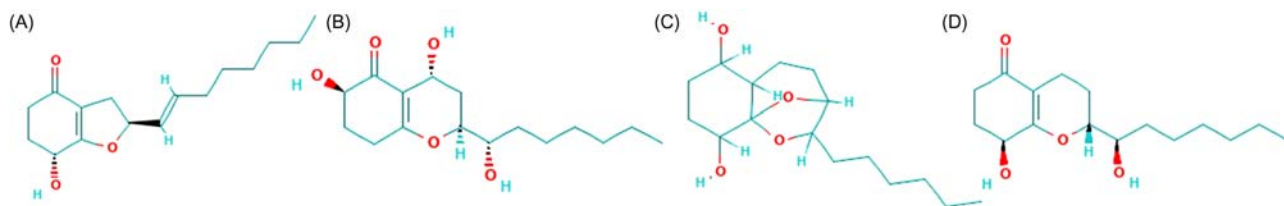


FIGURE 24.4 2D structure of Trichodermaketone C (A), Koninginin F (B), Koninginin A (C), Koninginin B (D) isolated from *Trichoderma Koningiopsis*.

24.3.3 Terpenes/terpenoids

About tens of thousands of terpenoids have been produced by micro-organisms and plants (Schmidt-Dannert, 2015) thus making this group, the most abundant natural product on earth (Zellinger et al., 2016). Branched five-carbon isoprenyl diphosphate intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) fuse repeatedly to synthesize all terpenes. Successive head-to-tail condensation of these C-5 units yields linear chain of prenyl diphosphate of varying lengths. These linear chains are the substrate for different key enzymes of terpene biosynthesis like prenyl transferases and cyclases as well as terpene synthases. Ultimately, these enzymes either transfer a prenyl chain to another molecule or trigger prenyl chain cyclization generating different isoprenoid-derived natural products (Christianson, 2008). *Trichoderma* spp. produce volatile terpenes that include tetracyclic diterpene harziandione, sesquiterpenes such as triterpene viridian, trichothecenes harzianum A and trichodermin (Stoppacher et al., 2010; Cardoza et al., 2011). Major terpenes produced by *Trichoderma* spp. are trichothecenes, trichodermin, sesquiterpenes, triterpenes, harzianum A and sterols (Cardoza et al., 2005).

24.3.3.1 Sesquiterpenes

Sesquiterpene synthases are accountable for the formation of more than 200 distinct compounds, thus a key enzyme in sesquiterpenes biosynthesis (Sivasithamparam and Ghisalberti, 1998). Sesquiterpenes are formed by fusion of dimethyl allyldiphosphate (DMAPP) with isopentenyl diphosphate (IPP) units via farnesyl diphosphate (farnesyl-PP) (Zeilinger and Schuhmacher, 2013). Sesquiterpenes synthesized by *Trichoderma* have inhibitory effects on bacteria, fungi and yeast along with neuroleptic activities (Berg et al., 2003, Bennett and Klich, 2003). Daucane sesquiterpenes, 3, 4 dihydroxycarotane and lignoren are the examples of sesquiterpenes having antimicrobial activities against *Bacillus subtilis*, *Sporobolomyces salmonicolor*, *Mycobacterium smagmetisa*, *Pseudomonas aeruginosa* and *Rhodotorula rubra* (Berg et al., 2004). Li et al. (2011) isolated three new acorane sesquiterpenes viz., 2 β -hydroxy-1 α -isopropyl-4 α ,8-dimethylspiro [4.5] dec-8-en-7-one, 1 α -isopropyl-4 α ,8-dimethylspiro [4.5] dec-8-ene-3 β ,7 α -diol and 1 α -isopropyl-4 α , 8-dimethylspiro [4.5] dec-8-ene-2 β ,7 α -diol from *Trichoderma* spp. (YMF1.02647). Three new sesquiterpenes, Trichodones A–C were isolated from the crude extract of an endophytic fungus i.e. *T. asperellum* (Dinga et al., 2012). Berg et al. (2004) isolated lignoren from *T. lignorum* HKI 0257, a new sesquiterpenoid metabolite with a moderate antibacterial and antifungal activity against *R. rubra*, *Mycobacterium smegmatis*, *Sporobolomyces salmonicolor*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.

24.3.3.2 Trichothecenes

Trichothecene term is derived from trichothecin, one of the first identified members of the family (Bennett and Klich, 2003). Apart from *Trichoderma* spp., trichothecenes are also produced by *Fusarium*, *Stachybotris* and *Myrothecium* (Degenkolb et al., 2008). Trichothecenes are closely related to sesquiterpenes, contain a 12, 13-epoxytrichothec-9-ene (EPT) with variable number of hydroxyl or acetoxy groups. The analogs of trichothecenes differ in terms of substituents attached to EPT (Proctor et al., 2018). In *Fusarium* spp., transport and regulatory proteins which are encoded by trichothecenes biosynthetic gene (*TRI*) has been identified as core clusters directly involved in its biosynthesis (Brown et al., 2004). Cardoza et al. (2011) described trichothecene biosynthesis and a *TRI* cluster in another trichothecene-producing genus, *Trichoderma*, although they differ remarkably from the larger *Fusarium* cluster in terms of gene content and organization. In *T. brevicompactum* and *T. arundinaceum*, Cardoza et al. (2011) identified and characterized *TRI* gene orthologues (*tri*). The gene content and organization in *tri* cluster was same for both *Trichoderma* spp. but differed from *Fusarium* spp. Based on sequence and functional analysis, it has been observed that unlike in *Fusarium* spp., *tri5* gene regulates the first step in trichothecene biosynthesis is located outside the cluster in both *Trichoderma* spp. (Cardoza et al., 2011). During course of evolution of *Fusarium* and *Trichoderma*, the clusters have undergone distinct changes, however some characteristics remained conserved. Harzianum A (HA) and trichodermin (Fig. 24.5A and B) are two common trichothecenes. Trichodermin was isolated from *T. viride* in 1964 by Godtfredsen and Vangedal, later

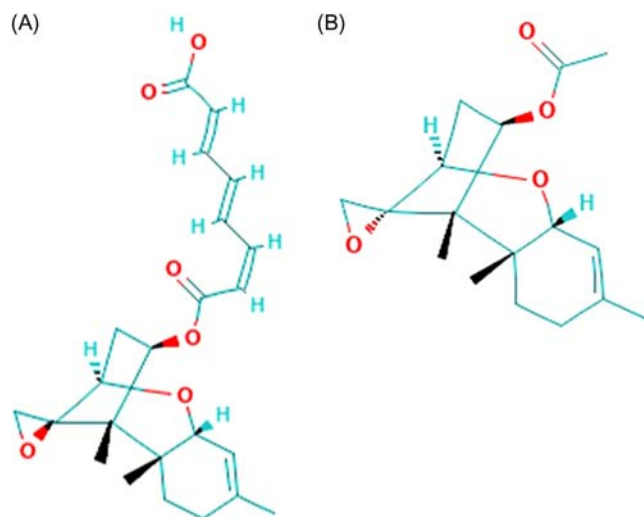
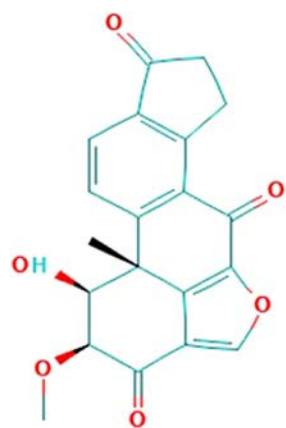


FIGURE 24.5 2D structure of Harzianum A (A), Trichodermin (B).

FIGURE 24.6 2D structure of viridin from *Trichoderma koningiopsis*.

Adams and Hanson in 1972 and Watts et al. in 1988 obtained this trichothecene from *T. polysporum*, *T. sporulosum* and *T. reesei*, respectively. Two *Trichoderma* spp. viz., *T. arundinaceum* and *T. brevicompactum* have seven genes- *tri6*, *tri14*, *tri10*, *tri12*, *tri3*, *tri4* and *tri11* in *tri* cluster (24,793 bp) that required for the biosynthesis of trichodermin. Shentu et al. (2018) described the effect of *tri4*, *tri3* or *tri11* disruption on trichodermin biosynthesis and expression of other genes in the *tri* cluster and first time reported the role of *tri4*, *tri11* or *tri3* in *T. brevicompactum*.

24.3.3.3 Viridin

Viridin (Fig. 24.6), discovered by Brian and McGowan in 1945, is a furano steroidal antibiotic with fungistatic and anticancerous properties first isolated from *T. viridae*. However, later it was also detected in other *Trichoderma* spp. such as *T. koningii* (Berestetskii et al., 1978) and *T. viride* (Golder and Watson, 1980). Viridin belongs to pentacyclic furanosteroids family has an electron deficient furan ring fused between C-4 and C-6 of the steroid framework (Hanson, 1995). Viridin is irreversibly reduced to viridol, a compound having herbicidal properties therefore engineering the viridin pathway by blocking viridin conversion to viridol would reduce the phytotoxicity and result in accumulation of antifungal viridian (Mukherjee et al., 2006; Andersson et al., 2010). Brian and McGowan (1945) and Ghisalberti (2002) reported the prevention of spores germination of *Stachybotrys atra*, *Colletotrichum lini*, *Botrytis allii*, *Aspergillus niger*, *Penicillium expansum* and *F. caeruleum* by viridin. Mukherjee et al. (2006) identified six genes including four cytochrome P450 genes, one terpene cyclase and one O-methyl transferase which were similar to those involved in secondary metabolism of other fungi by comparing the transcriptional pattern of wild type and a secondary metabolite deficient *T. virens* mutant. Three cytochrome P450 genes and one terpene cyclase are located as clusters. Mukherjee et al. (2006) advocated the association of this cluster in production of viridin. In 2018, Bansal et al. reported complete biosynthetic gene cluster of 21 genes in *T. virens* playing role in viridin biosynthesis. This gene cluster contains six cytochrome P450 monooxygenases and oxidoreductases, and MFS transporter but lacks a transcription factor.

24.3.4 Anthraquinone

Many *Trichoderma* spp. have been reported to produce anthraquinone, a largest group of natural quinones. Anthraquinones are principal group of secondary metabolites present in bacteria, fungi, lichens and higher plants as well. Besides being antifungal, antiviral and anticancer agents, anthraquinones also serve as laxatives, diuretics, phytoestrogens and immune stimulators. Slater et al. (1967) isolated pachybasin, chrysophanol and emodin produced by a wild strain of *T. viride* in soil. Since then many other workers isolated anthraquinones from different *Trichoderma* spp. Takuo et al. (1983) isolated trichodermaol from combined culture of *Trichoderma* spp. with *F. solani* or *F. oxysporum* and elucidated its structure by spectroscopic and X-ray crystallographic analysis. Liu et al. (2007) isolated two anthraquinone derivatives viz., chrysophanol and pachybasin through a silica column chromatography from *T. harzianum* ETS 323 grown on sugarcane bagasse solid medium incubated at room temperature without rotation. *T. polysporum* produced previously isolated pachybasin, chrysophanol and emodin while in contact with *Fomes annosus* (Donnelly and Sheridan, 1986). Among six anthraquinone derivatives isolated from *T. harzianum* strain Th-R16 (Pachybasin, chrysophanol, emodin, 1, 5-dihydroxy-3-hydroxymethyl-9, 10-anthraquinone, 1, 7-dihydroxy-3-hydroxymethyl-9, 10-anthraquinone and ω -hydroxypachybasin) (Fig. 24.7), four have been identified for the first time by Liu et al. (2009). Harzianumones A and B, two hydroxyanthraquinones have been isolated from *T. harzianum* (Shi et al., 2018). Anthraquinones have been reported to have antimicrobial activities against *S. aureus*, *R. solani*, *B. cinerea*, *P. aeruginosa*, *Bacillus cereus* and *Escherichia coli* (Liu et al., 2007).

24.4 Lytic enzymes of *Trichoderma* Spp

Apart from production of secondary metabolites with antiproliferative and antimicrobial activities, *Trichoderma* spp. is known to produce extracellular hydrolytic enzymes which aid in the localized lysis of cell wall of phytopathogenic fungi during the course of mycoparasitism. Though these lytic enzymes are not just involved in mycoparasitism but also play a role in cell wall remodeling and recycling during active fungal growth as well as during ageing and autolysis (Gruber and Seiboth, 2012). The lytic tool box of *Trichoderma* spp. principally contains chitinases (Endo, exochitinases and N-acetyl- β -D-glucosaminidase), β -1, 3-glucanases (Endo and exo- β -1, 3-Glucanases) and proteases (Markovich and Kononova, 2003). Chitinases/chitin degrading enzymes are the representative of *Trichoderma* produced lytic enzymes. Chitin is a natural polysaccharide found in abundance as structural constituent of most fungal cell walls, insect's exoskeleton. Chitinases are classified into three classes viz., endochitinases (release chitotetraose, chitotriose, and chitobiose following the random internal bond cleavage of chitin fibril), exochitinase (also called as chitobiosidase) catalyzes the diacetylchitobiose release without oligo- or monosaccharides formation and β -N-Acetylhexosaminidases (also termed as N-acetyl- β -D-glucosaminidases and chitobiase) catalyze the release of GlcNAc monomers following the cleavage of chitin polymer in an exo-type fashion. Most of the fungal chitinases belong to glycoside hydrolases (GHs) 18 family. β -(1,3)-glucan and 1,4- β -D-glucan (cellulose) are other predominant fibrillar polymer in fungal cell wall after chitin which is degraded by glucanases. Apart from chitinases and glucanases, proteases are also indispensable for

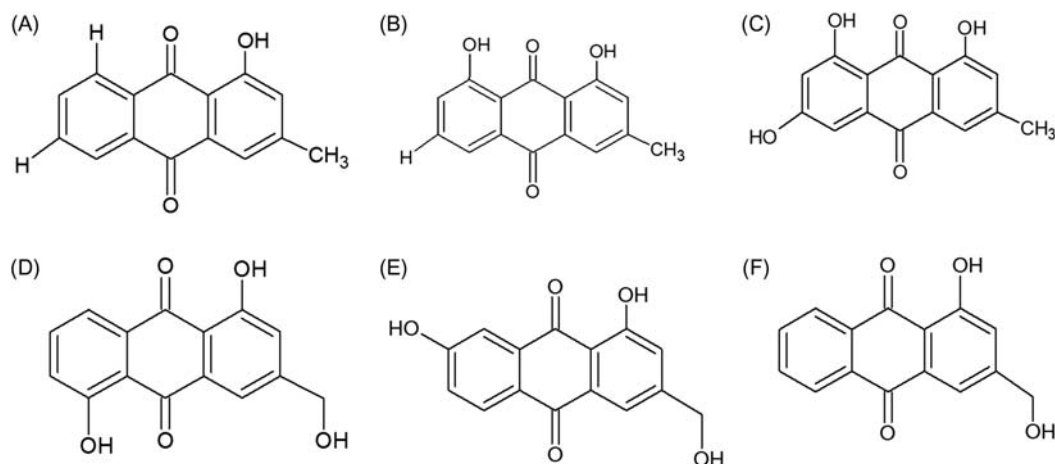


FIGURE 24.7 Chemical structures of Pachybasin (A), Chrysophanol (B), Emodin (C), 1,5-Dihydroxy-3-Hydroxymethyl-9,10-Anthraquinone (D), 1,7-Dihydroxy-3-Hydroxymethyl-9,10-Anthraquinone (E) and ω -Hydroxypachybasin (F).

fungal cell wall degradation. Workers across the world have explored the possibility of transgenic plants expressing the *Trichoderma* genes producing hydrolytic enzymes for managing fungal disease, however in some cases along with reduced disease level, the plant growth was also affected (Fig. 24.8).

24.5 Beneficial effects of *Trichoderma* spp. on plants

Trichoderma spp. has been designated as significant fungus for agriculture and environment (Waghunde et al., 2016). The genus is not just known for its application in plant disease management but also to elevate the plant growth and strengthen the plant defense mechanism (Harman et al., 2004; Schuster and Schmoll, 2010; Daguerre et al., 2014).

- i. Plant disease management:** The potential of this wonder fungus in plant disease management is now well established. A number of *Trichoderma* based formulations have been registered (Table 24.2) and are being recommended for the management of various soil borne pathogens (Woo et al., 2014).

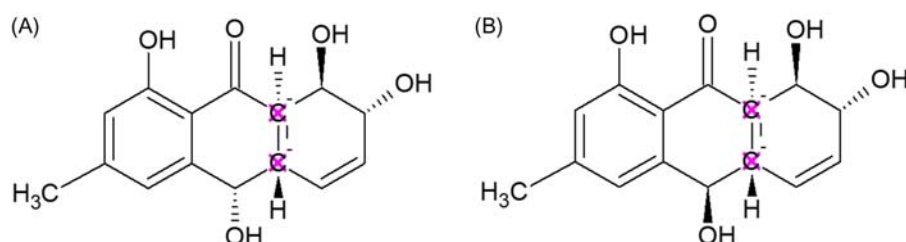


FIGURE 24.8 2D structure of Harzianumnes A and B.

TABLE 24.2 Some registered commercially available *Trichoderma* based formulations used against plant diseases in India.

S. No.	Product name	Active ingredient	Targets
1.	ANOKA	<i>T. viride</i> , <i>Pseudomonas fluorescens</i>	Nematodes, Root rot, collar rot and wilt causing fungi
2.	Agrigold, Trichogold	<i>T. viride</i>	Downy and powdery mildews, Wilts, root and stem rot causing fungi, White rusts and smuts, Blights/leaf spots causing fungi
3.	Bio Protectore	<i>T. viride</i>	Wilts, root rot, brown rot, damping off, charcoal rot causing fungi and other soil borne fungi
4.	BioAgent ST-9	<i>T. viride</i>	<i>Rhizoctonia</i> , <i>Fusarium</i> , <i>Phytophthora</i> , <i>Pythium</i> , <i>Sclerotium</i> etc
5.	Bioderma H	<i>T. harzianum</i>	Root (<i>Phytophthora</i> , <i>Pythium</i> , <i>Fusarium</i>) and foliar (<i>Alternaria</i> , <i>Ascochyta</i> , <i>Collectotrichum</i> , <i>Macrophomina</i>) pathogens and <i>Ralstonia</i>
6.	Bio-Shield	<i>T. viride</i>	<i>Pythium</i> , <i>Phytophthora</i> , <i>Rhizoctonia</i> , <i>Sclerotium</i>
7.	Bioveer	<i>T. viride</i>	Damping off, root rot, foot rot, stem rot, collar rot, wilt causing fungi, Bacterial leaf blight (BLB) of rice
8.	Ecosom-TH	<i>T. harzianum</i> IIHR-Th-2	<i>Fusarium</i> spp, <i>Rhizoctonia</i> spp, <i>Pythium</i> spp. and <i>Alternaria</i> spp.
9.	Fertimax	<i>T. viride</i> + <i>T. harzianum</i>	Wilts, damping off, root rots, brown rot, Charcoal rot and other soil borne fungi
10.	Gliocladin (Trichodermin T, Z)	<i>T. harzianum</i>	<i>Fusarium</i> , <i>Verticillium</i>
11.	Neemoderma	<i>T. viride</i> / <i>T. harzianum</i>	<i>Colletotrichum</i> , <i>Alternaria</i> , <i>Cercospora</i> , <i>Helminthosporium</i> , <i>Rhizoctonia</i> , <i>Fusarium</i> , <i>Sclerotium</i> , <i>Verticillium</i>
12.	Niprot	<i>T. viride</i> or <i>T. harzianum</i>	Root knot nematode, Root, Rhizome foot and collar rot, <i>Pythium</i> spp.
13.	Shakti-AG	<i>T. viride</i> , <i>T. harzianum</i> and <i>Paecilomyces lilacinus</i>	Nematode, Soil borne fungi
14.	Tricho Shield Combat	<i>T. viride</i>	Root rot, Damping off and Wilt causing fungi
15.	Trichoguard	<i>T. viride</i>	<i>Fusarium</i> , <i>Phytophthora</i> , <i>Rhizoctonia</i> , <i>Pythium</i> and <i>Botrytis</i> fruit rot

- ii. **Plant growth promotion:** The ability to promote the growth, development and productivity of various vegetable, field, ornamental and other horticultural crops by *Trichoderma* either through application in soil/growing media or seed/seedling treatment have been reported since many years. Though there are many reports on the positive effect of *Trichoderma* spp. on plant growth, however some researchers have also observed its negative effect on the plant growth promotion (Stewart and Hill, 2014). This ability of *Trichoderma* is not species dependent as considerable amount of variation in the extent of growth promotion is found among different species and even among different isolates of same species (Ousley et al., 1993; Rabeendran et al., 2000). Moreover, the bioactivity increases when mixture of *Trichoderma* spp. or with other microbes like *Glomus mosseae*, *Bacillus subtilis* are used (Rudresh et al., 2005). The mechanism which makes this genus as a plant growth promotor includes suppression of major pathogens which prevent the plant to achieve its highest yield potential, rhizosphere competence and endophytic activity, mineral solubilization and enhanced nutrient uptake, production of phytohormones, better root architecture.
- iii. **Abiotic stress relief:** A lot of studies have been undertaken in applications of *Trichoderma* spp. in biotic stress management, however its potential in relieving the abiotic stress has not been exploited (Zaidi et al., 2014). *Trichoderma* can also promote growth and confer resistance to plants under abiotic stresses like drought, soil salinity, heat stress, pH, osmotic stress etc. Under stress conditions plants accumulate reactive oxygen species (ROS) at a toxic level. As a result of plant root colonization by *Trichoderma* spp., compounds like phytoalexins and phenol are accumulated with in the plants which ultimately act as scavengers to these ROS thereby protecting the plant from abiotic stresses.
- iv. **Plant immunity:** A plant inoculated with *Trichoderma* spp. has shown improved resistance level both locally and systemically mainly due to the accumulation of antimicrobial compounds, volatile organic compounds and phytoalexins as a resultant of complex signal transduction networks.

24.6 Conclusion

It is very evident that *Trichoderma* spp. is a wonder fungus, a prolific producer of secondary metabolites and hydrolytic enzymes having vast biocontrol potential against pathogens, known to induce resistance in plants and enhance their growth and development. Since the discovery of Gliotoxin, the first antimicrobial compound in 1934 by Weindling, an array of secondary metabolites produced by *Trichoderma* spp. were identified and characterized. However, still there are multitude of unidentified and uncharacterized metabolites with unknown functions and structures. The advancements in the molecular biology technologies have resulted in availability of different *Trichoderma* spp. genome. The comparative analysis of *Trichoderma* spp. genomes has led to the identification of new secondary metabolites and characterization of plentitude of gene clusters involved in the production of these metabolites. Furthermore, genome mining strategies have been employed by different workers to identify more gene clusters involved in secondary metabolism of the fungus. Many transcription factors including LaeA and velvet complex proteins have been identified to play crucial role in secondary metabolism regulation of fungi though the underlying mechanism is not very well understood. Moreover, the identification of various ecological and physiological factors that activates the gene clusters involved in secondary metabolite regulation is yet another challenging goal. Integration of high throughput genomics, transcriptomics, proteomics and metabolomics to get an in depth understanding of regulation and biosynthetic mechanism, different biotic and abiotic factors which induces the production of *Trichoderma* metabolites, identification and structural characterization of genes involved in secondary metabolism and the mechanism of *Trichoderma*-plant-pathogen interaction is required though problem of big data and its integration also comes with the multiomics approach. All detailed knowledge generated will ultimately enable the development of tailor-made *Trichoderma* strains capable of producing adequate amount of significant metabolites that can be further employed in the sustainable management of stubborn plant diseases.

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Chapter 25

Endophytic fungi: positive association with plants

Juliana Oliveira de Carvalho^{a,1}, Valquiria Broll^{b,1}, Anne Helene Souza Martinelli^c and Fernanda Cortez Lopes^{a,d}

^aGraduate Program in Cell and Molecular Biology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, ^bLaboratory of Bioinorganic Chemistry, Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy, ^cDepartment of Biophysics & Department of Molecular Biology and Biotechnology, Biosciences Institute (IB), Federal University of Rio Grande do Sul, Porto Alegre, Brazil, ^dCenter for Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

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25.1 Introduction

Plants interact with a vast microbial community. These microorganisms can be associated with plant surface as well as established in a kind of microflora. Plant-microbial associations have an interspecific interaction, which can be positive or negative such as cooperation or competition, respectively. To classify the type of plant-microorganism interaction some authors use symbols, +1 to define the interaction as beneficial effect for at least one of the species in study, 0 as neutral effect and −1 is classified as negative effect for one of the participants (Burkholder, 1954; Martin and Schwab, 2012; Singh et al., 2017).

There is a great variety of interspecific plant-microorganism interaction. Neutral, where both organisms that do not impact each other, positive or negatively. Positive interaction can be divided into: Mutualism, where both members are positively benefited by their interaction (+1; +1); Commensalism one integrant is favored while its partner is not affected (+1;0) or Synergism, which is a beneficial interaction for both individuals (+1; +1); but in that case, both organisms can live independently (Atlas and Bartha, 1998). There are some negative interactions also, they could be divided into: Antagonism, when a species affects direct or indirectly the growth of other species (+1; −1); Parasitism, a condition where one of the organisms involved lives inside the other, a host-parasite relationship is settled down (+1; −1); Predation is a relationship between two individuals as predator-prey, where the first attacks its prey, feeding on it (+1; −1); Competition is a relationship where one of the organisms will survive dominating its competitor (−1; −1) (Singh et al., 2017).

Fungi, bacteria, archaea and protists can be found in the external environment at the surface of plants (epiphytes) or in a microenvironment - inside plant tissues (endophytes) (Campisano et al., 2015; Salazar-Cerezo et al., 2018). Microorganism-plant interaction is essential on plant development, resistance to diseases and response to biotic and abiotic events (Salazar-Cerezo et al., 2018; Strobel, 2018). Endophyte is a term that comes from a combination of two

1. These authors contributed equally to this work.

Greek words, “endon” (within) and “phyton” (plant) (Shah et al., 2017). In order to be characterized as an endophytic microorganism, it should live at least part of its life cycle inside a plant and be active in the maintenance of the macroecosystem (Shah et al., 2017). Endophytic microorganisms should pass through some biological and physical barriers and avoid recognition by its host (Yan et al., 2018).

Fungi that live in symbiosis inside its host can present different types of interaction, which could be positive as commensalism, mutualism or even negative as antagonism. To be considered as an endophytic fungus is important that the interaction is not harmful to the host, and if the fungus is a pathogen, the illness symptoms should not be detected (Strobel, 2018). To avoid symptomatic pathogenicity, the fungus should avoid recognition by the host and it can be provided if there is a balance between defense mechanism and virulence factors but not only. To be able to penetrate the plant barriers the fungi needs to pre-adapt itself in a very complex system of co-evolution between plant-endophyte (Kusari et al., 2012). Historically, the oldest endophytic interaction between a fungus and its plant host documented is the arbuscular mycorrhiza (AM). That root-fungus mutualistic interaction turned to be crucial to the terrestrial life of early plants allowing soil phytoremediation (Depresseux, 1979; Redecker et al., 2000).

Endophytic fungi are classified into two groups: clavicipitaceous endophytes and non-clavicipitaceous endophytes, C- endophytes and NC- endophytes, respectively (Fig. 25.1). C- endophytes are phylogenetically related to the *Clavicipitaceus* species which present the ability to colonize grasses with a vertical transmission over generations, via seed infection. NC- endophytes is composed by a larger group able to colonize tissues of vascular and nonvascular plants. The nonvascular group includes bryophytes, whereas the vascular is composed by pteridophytes, conifers and angiosperms. NC-endophytes are sub-grouped into three categories based on colonization pattern, biodiversity and ecological effect on plant and mechanism of its propagation over generations. NC-endophyte type 1 is present in all the plants being transmitted over generations vertically as well as horizontally, instead type 2 is found only in aerial tissues causing a localized infection being transmitted horizontally over generations. Type 3 NC-endophytes subgroup is able to colonize only under-ground tissue (roots) causing an extensive tissue colonization (Rodriguez et al., 2009).

Fungi population and its abundance inside plant tissues is not static, it is closely linked to biotic and abiotic factors. Abiotic stress is an environmental situation related to aridity, extreme and unstable temperatures, salinity, chemical toxicity, soil pH, smog, and other, i.e., all vital factors for the plant, affecting its growth, production and propagation. The endophytic fungi communities can play a very important role processing some heavy metals that are toxic for plants; they can act reducing the environmental stress level by chelating some toxic metals due to its vast mechanism of tolerance (Deng and Cao, 2017; Shah et al., 2017). Once the interaction is established, fungi can strongly collaborate to help the plant to overcome stressful events (Deng and Cao, 2017). Living organisms such as insects, micro-organisms and

Phyllosphere

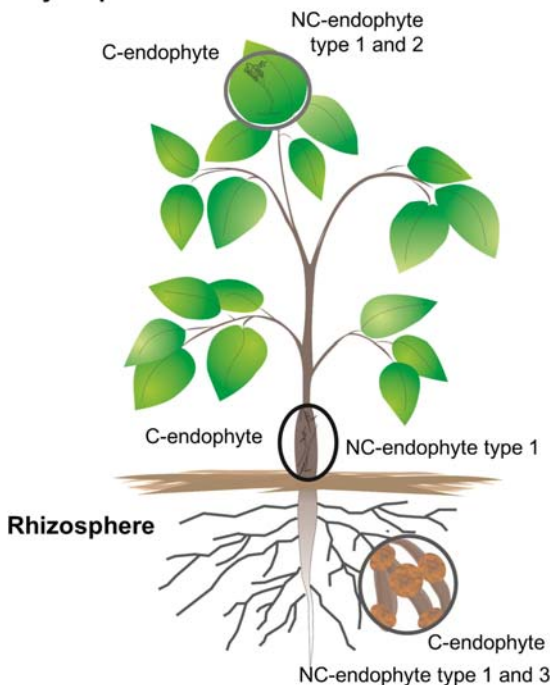


FIGURE 25.1 Endophytic fungi: classification and localization in the plant.

weeds, can inhibit plant growth, proliferation and productivity by competition or even causing diseases. In that case, some endophytic fungi can express some antagonistic compounds against pests, helping the plant to overcome its competitors (Jaber and Ownley, 2018). This chapter has the intent of providing a brief recent overview of mainly endophytic fungi and their positive interaction with host plants, considering their influence on abiotic and biotic stresses.

25.2 Endophytic fungi diversity and host plants localization

Endophytic fungi lives in internal plant tissues such as leaves, stems, roots, fruits, seeds and others parts, without causing harmful or symptoms to the host (Sunitha et al., 2013; Fouda et al., 2015). Nowadays there are several works about endophytes, which highlight their importance due to their immense diversity and numerous benefits brought to the host. In Table 25.1, we summarized some examples of endophytic fungi and their localization in the plant host.

Studies demonstrated that the positive interaction of host plant with mutualistic fungal endophytes could increase plant tolerance to biotic and abiotic stresses. *Neotyphodium* spp. isolated from grasses, for example, was reported to be involved in the alteration of the antioxidant activity in plant hosts responding to drought (Schulthess and Faeth, 1998; Schardl et al., 2004; Hamilton and Bauerle, 2012).

In the same line, the endophytic fungus *Piriformospora indica*, frequently found in roots, are described as a beneficial fungal that promotes plant growth, improving host tolerance to abiotic stress, such as salinity (Baltruschat et al., 2008) (for a review see Oelmüller et al., 2009).

On the other hand, fungi traditionally known as insect pathogens, like *Beauveria*, *Metarhizium*, *Cladosporium*, *Clonostachys* and *Isaria* species can be artificially inoculated and act as plant endophytes, bringing many benefits to the plant such as disease antagonism and also increasing plant growth (Vega, 2008; Blackwell et al., 2009) (see Table 25.1). Some of these entomopathogenic fungi are classified as non-clavicipitaceous, based on horizontally transmission of these fungi (Rodriguez et al., 2009). As an example of this positive interaction, Lopez and co-workers demonstrated the effects of *Beauveria bassiana* and *Purpureocillium lilacinum* in *G. hirsutum*. These endophytes were inoculated in seeds of cotton and were able to promote plant growth. They increased the dry biomass, the number of nodes and reproductive tissues, as well as, inhibited the development of a crop pest bollworm (*Helicoverpa zea*) (Lopez and Sword, 2015). Khan et al. (2012a,b) showed that *Metarhizium anisopliae* LHL07, isolated from *G. max* roots, could be used as endophytic fungi interacting with *G. max* beans through artificial inoculation and improving the quality and quantity of plants under salt stress conditions (Khan et al., 2012a,b) (see Table 25.1).

Hassan and co-workers isolated *Penicillium chrysogenum* and *P. crustosum* from leaves of the medicinal plant *Teucrium polium* and demonstrated that these endophytes produced indole acetic acid (IAA), ammonia, and enzymes with antimicrobial activities, which are able to promote plant growth and its maintenance (Hassan, 2017). Golparvan and co-workers studied the effect of the endophytic fungus *Aspergillus* sp., isolated from the medicinal plant *Lippia citriodora*. It was demonstrated that the endophyte promoted the plant development, increasing the number of leafs and other tissues (Golparyan et al., 2018).

The interactions between endophytic fungi and the host plants are yet to be fully understood (Aly et al., 2011). It is believed that the plant-endophyte mutualism started with its co-evolution and this relationship was modeled by environment and physiological characteristics (Kogel et al., 2006; Giménez et al., 2007). In the next sections we will explain in more details the action of these endophytic fungi helping the plant to combat abiotic and biotic stresses (Fig. 25.2).

TABLE 25.1 Examples of Endophytic fungi and their localization in host plants.

Endophyte fungi	Localization	Host plant	Reference
<i>Neotyphodium starrii</i>	Grass	<i>Festuca arizonica</i>	(Schulthess and Faeth, 1998)
<i>Lecanicillium lecanii</i>	Leaves	<i>Gossypium hirsutum</i> L.	(McGee, 2002)
<i>Metarhizium anisopliae</i> LHL07	Roots	<i>Glycine max</i>	(Khan et al., 2012a,b)
<i>Phoma glomerata</i> and <i>Penicillium</i> sp.	Roots	<i>Cucumis sativus</i>	(Waqas et al., 2012b)
<i>Lasioidiplodia</i> sp.	Stem	<i>Portulaca oleracea</i>	(Deng et al., 2014)
<i>Penicillium chrysogenum</i> and <i>P. crustosum</i>	Leaves	<i>Teucrium polium</i>	(Hassan, 2017)
<i>Phialemonium inflatum</i> (TAMU490)	Leaves	<i>G. hirsutum</i> L.	(Sword et al., 2017)
<i>Aspergillus</i> sp. F14	Stem	<i>Lippia citriodora</i>	(Golparyan et al., 2018)

25.3 Plant protection against abiotic stress

The availability of water, carbon, nutrients, minerals, the intensity and quality of the light are the main requirements for the development of a plant. Due to their limited mobility, the extreme variations of these abiotic factors and the attack of pathogens, cause physiological and biochemical consequences for all the plants (Lata et al., 2018). The loss of homeostasis raises the importance of the relationship between plant-endophyte. Studies have shown that the interaction between endophytic fungi and plants brings benefits mainly for the host, with tolerance to multiples abiotic stresses (Khan et al., 2013b; Kavroulakis et al., 2018; Keppanan et al., 2018) and promoting plant growth (Hamayun et al., 2012; Latif et al., 2013; Li et al., 2017). In this session will be discussed some plant physiological adaptations, that come from symbiosis with the fungus, when the plant is under abiotic stress.

25.3.1 Oxidative stress modulation

In superior plants and algae, reactive oxygen species (ROS) are produced continuously as subproducts of a large number of metabolic pathways (photosynthesis, photorespiration and respiration) localized in different cell compartments as chloroplasts, peroxisomes and mitochondria (Halliwell, 2006; del Luís et al., 2006; Navrot et al., 2007). The plant's cell redox homeostasis is formed by ROS, the functionality of the antioxidant enzymes and the non-enzymatic antioxidants (Gill and Tuteja, 2010).

Different physiological alterations occur in plants under stress conditions, and sometimes bring intracellular and functional disturbances in the tissue. ROS accumulation induced by these conditions disturbs the equilibrium between production and elimination of ROS (Trchounian et al., 2016). Some studies have been shown the association with endophytic fungi to restore the redox homeostasis in the plant, reducing ROS levels and allowing the development of the host in environmental stress conditions (Hamilton et al., 2012; Hamayun et al., 2017; He et al., 2017).

Antioxidant activity and ROS production have been shown to be significant during colonization by endophytic fungi and frequently positive for the plant (Hamilton et al., 2012). Endophytic fungi induce the production of many compounds and antioxidant enzymes, for example, *Aspergillus flavus* CHS1 (Hamilton and Bauerle, 2012). The fungus promoted growth in salt-stressed soybeans by controlling their antioxidant enzymes, such as peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT). These enzymes are capable of catalyzing the detoxification of hydrogen peroxide and superoxide (Trchounian et al., 2016). Besides antioxidant enzymes, phenolic acids, flavonoids, carotenoids, tanines, phenolic terpenoids and glutathione also show antioxidant action and are regulated by endophytic fungi. These non-enzymatic compounds can neutralize ROS by accepting electrons (Huang et al., 2007b; Hamilton and Bauerle, 2012; Hamilton et al., 2012).

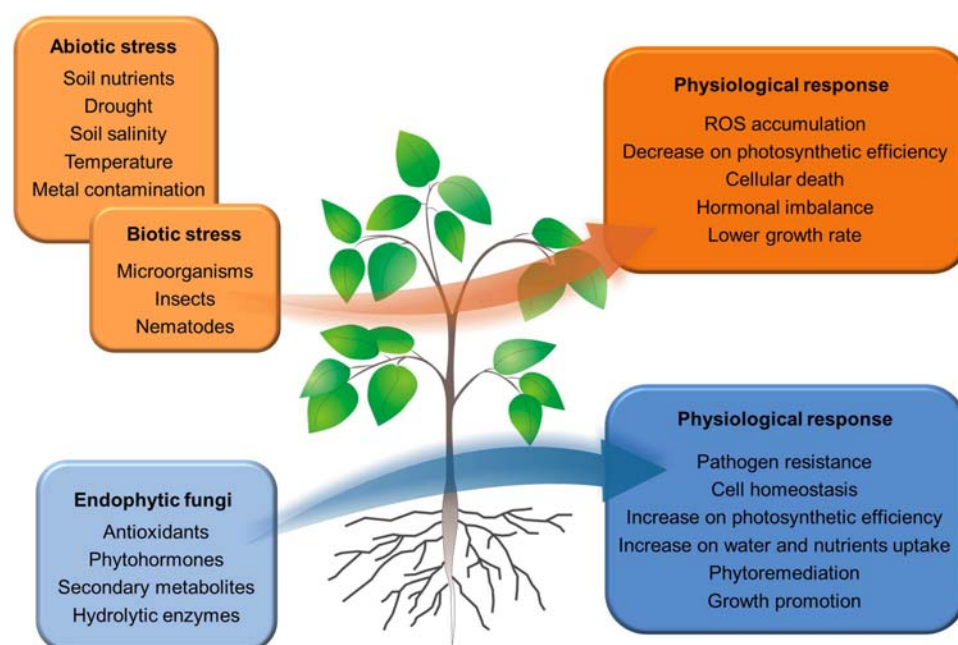


FIGURE 25.2 Biotic and abiotic factors affecting plants and their interaction with Endophytic fungi.

Neotyphodium (Hamilton and Bauerle, 2012), *Alternaria* spp., *Paecilomyces formosus* (Latif et al., 2012), *Phoma glomerata* LWL2, *Penicillium* spp. (Waqas et al., 2012b), *Trichoderma harzianum* (Azad and Kaminskyj, 2015) and *Exophiala pisciphila* (He et al., 2017) are also examples of endophytic fungi that help against ROS, and promote growth of host plants under abiotic stress (such as heavy metals, drought, salt and high temperatures).

25.3.2 Alteration of phytohormonal homeostasis

Phytohormones are organic compounds involved in growth, physiological processes and plant defense mechanisms. Endophytic fungi that are associated with plants can effectively change the phytohormonal homeostasis and manipulate the physiological alterations under environmental stress (Egamberdieva et al., 2017). Gibberellic acid (GA) is a phytohormone that controls plant's growth and promotes flowering, stem elongation, seed germination and ripening (Taiz et al., 2017). Many endophytic fungi present active GAs biosynthetic pathway, contributing for a better yield of the host plant, like *Penicillium* sp., *Phoma glomerata* (Waqas et al., 2012b) and *Porostereum spadiceum* (Hamayun et al., 2017). In filtered culture of the endophytic fungus *P. spadiceum*, different bioactivated GAs were found, including species with an exponential role in the promotion of the plant's growth, GA1, GA3 and GA4 types. Soybean seedlings colonized with this fungus presented higher production of GA1 and GA4 and increased biomass when compared with the control that was under salt stress. *P. spadiceum* induced seedling growth under stress not just by the rising levels of GAs, but also by inducing abscisic acid (ABA) catabolism and, consequently, by controlling better water absorption and/or retention (Hamayun et al., 2017). In other studies, ABA response to GA levels was almost similar in every abiotic stress condition, being it salt, drought, heat or heavy metals (Khan et al., 2011; Latif et al., 2011b).

ABA regulates several pathways of stress signaling and promotes seed dormancy. Plant's response to stress is reducing its growth by increasing ABA and lowering GA (Egamberdieva et al., 2017). However, in normal conditions, GA has an antagonistic role to ABA and favors germination. In the germination of the seed, the expression of genes is induced by GA signalization, synthesizing hydrolytic enzymes that are responsible for the endosperm nutrient reservoir degradation (Waqas et al., 2012a). In that process, the endophytic fungus associated with seeds degrades the endosperm and provides free carbon to the seedlings, favoring germination and vigor (Jerry, 2011). An example is the *Penicillium funiculosum*, this endophytic fungus caused increased speed of germination in soybean seeds under salt stress due to the increase of GA4 (Latif et al., 2011a). The high concentration of ABA during this process is also correlated with the leaf expansion inhibition and with the development of aerial parts in other species. Although, colonized plants with different endophytic fungi present bigger leaf area and better photosynthetic efficiency (Latif et al., 2011a; Redman et al., 2011; Hamayun et al., 2017).

Many physiological processes are influenced by the homeostasis of IAA, especially cell differentiation, germination, vascular tissue formation, growth, pigmentation, photosynthesis and root elongation (Zhao, 2018). Some endophytes, especially those established in the roots, accelerate the growth and development of the plant through the production of IAA (Deng and Cao, 2017; Li et al., 2017). IAA produced by endophytic fungus is responsible for stimulating root growth, nitrogen absorption, photosynthesis increase and promote the growth of the host during many abiotic stresses (Latif et al., 2011a; Li et al., 2017; Rozp et al., 2018). *Phoma glomerata* LWL2 and *Penicillium* spp., for example, produce bioactive auxin that promotes host's growth, increasing essential nutrients assimilation, such as calcium, potassium and magnesium under salt stress (Waqas et al., 2012b).

Salicylic acid (AS) and jasmonic acid (JA) also play an important role in plant defense. These may act synergistically or antagonistically with each other (Waqas et al., 2012b). Increased levels of JA were observed in the growth of several plants exposed to abiotic stresses. For example, plants that grow in association with the endophytic fungus *P. glomerata* reduced JA and increased SA contents, which may be associated with the mitigation of saline and water stresses caused by this fungus (Waqas et al., 2012b). Endophyte *Aspergillus fumigatus* sp. LH02 can regulate soybean plant growth during saline stress and increase endogenous JA levels (Latif et al., 2011a). These different studies show that fungal endophytic symbiosis provides different mechanisms of JA/SA responses.

25.3.3 Phytoremediation and heavy metals tolerance

Metal toxicity has high impact in plants and affects the ecosystem where the plant is found. Some metals, including copper, manganese, iron, cobalt, zinc and chromium are essential for the plant's metabolism, but in residual quantities. Plants growing in contaminated areas by metals have a tendency of accumulation of them in their cells. These plants normally present an altered metabolism, reducing their growth rates and biomass production (Kalaivanan and Ganeshamurthy, 2016).

Phytoremediation is based on the natural ability of plants to extract compounds from the environment (Khan and Doty, 2011). The success of phytoremediation is strongly determined by the growth and development of the plant and by the rate of accumulation of heavy metals in its tissue. Some phytoremediation plants adapt and develop very well in environments massively contaminated by heavy metals, but other plants may have limitations on their phytoremediation capacity and may suffer phytotoxicity due to slow degradation and evaporation of contaminants (Glick, 2003).

Endophytic fungus shows potential to enhance the phytoremediation and to fight against the phytotoxicity caused by the heavy metals in the soil. Many metal hyperaccumulating plants are associated with endophytic fungi, as *Trichoderma*, *Fusarium*, *Aspergillus* and *Penicillium* species (Deng et al., 2014). *Penicillium* spp. and *Trichoderma* spp. are the most frequently isolated and used to neutralize metal stress, caused by Ni^{2+} , Cd^{2+} , Cu^+ , Zn^{2+} and Pb^{2+} (Khan et al., 2013a; Babu et al., 2014; Hiruma et al., 2018).

Many of these fungi can survive and grow in environments highly contaminated with toxic metals. Sequestering and precipitation of the extracellular metal, binding the metal to the cell wall, intracellular complexation, compartmentalization and volatilization, allow these fungi to adapt and tolerate the toxic environment (Fomina et al., 2005). The endophytic fungus of roots, such as dark-septate endophytes, induce heavy metal tolerance of the plants by enhancing the expression of metal transporters associated with the metal influx, efflux and distribution inside the plant, by improving the antioxidative system and phytohormones production (Wang et al., 2016; He et al., 2017; Hiruma et al., 2018; Rozp et al., 2018).

25.4 Plant protection against biotic stress

The host plants are responsible to provide nutrients for the endophytes and also are important shelter to these microorganisms, while fungi secrete bioactive compounds to improve plant's development, as we mentioned in the last section, and also increase competitiveness in its natural habitat (Sun et al., 2014) that will be discussed in this section. Nowadays, many studies have been conducted, on the isolation and screening of active metabolites produced by endophytic fungi, mainly due to their secondary metabolism. Although, the first discovery of these fungi occurred more than a century ago, they were not enough studied until the recognition of their importance in pharmaceutical and ecology fields (Gunatilaka, 2006; Li et al., 2018). It is believed that each plant (around 300,000 plant species) can be a host to one or more endophytes (can reach hundreds of species and include endophytic bacteria, that will not be discussed in this chapter), increasing drastically the diversity of compounds that can be produced by these microorganisms (Strobel and Daisy, 2003; Huang et al., 2007a).

Endophytic fungi produce bioactive compounds including alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenols and lactones, that are very important to the pharmaceutical industry and also to agriculture (Zhang et al., 2006; Yu et al., 2010). These compounds showed several pharmacological activities such as antiviral, antibiotic, anticancer, antidiabetic and immunosuppressive. These fungi metabolites can be used as drug prototypal, due to the importance of these biological activities against some common human diseases (Strobel and Daisy, 2003; Gutierrez et al., 2012). Moreover, recent studies on hydrolytic enzymes, produced by endophytic fungi, showed their capacity on the control of plant pathogens (Shaikh and Sayyed, 2015; Shaikh et al., 2016). Enzymes such as chitinases, glucanases, proteases and cellulases are able to degrade structural pathogens constituents, including chitin, proteins, cellulose, hemicellulose and also DNA. Plant pathogens include fungi, bacteria, insects and nematodes and they have these target compounds in their cells (Hp and Rz, 2016).

It is important to highlight that endophytes could provide plants with these biologically active metabolites, thus contributing to their defense. They are also able to transfer the corresponding genes, through horizontal transfer, to the plant genome or vice versa (Wink, 2008). One important example is the discovery of the paclitaxel (Taxol)-producing endophytic fungus *Taxomyces andreanae*. This fungus had been isolated from the pacific yew *Taxus brevifolia*. Paclitaxel is an important drug used to the anticancer treatment and was firstly isolated from the plant *T. brevifolia* in 1967, evidencing genetic material transfer between these two species (Stierle et al., 1993; Stierle et al., 1995).

It is known that plants without endophytes would be more vulnerable to several types of stresses and to pest attacks (Leitao and Enguita, 2016; Suman et al., 2016; Brader et al., 2017). In this section, we will bring some examples of endophytic fungi that can produce insecticidal and antifungal compounds to protect plants against these pests.

25.4.1 Insecticidal properties

Fungal endophytes protect host plants against herbivores, acting as entomopathogenic agents. They cause infections in lepidopterous larvae, aphids, thrips, and other cosmopolitan insects, that are extremely harmful to agriculture (Arnold

and Lewis, 2005; Jallow et al., 2008; Akutse et al., 2014). Many fungi are considered entomopathogens such as *Beauveria basiana*, *Isaria farinosa*, *Cladosporium* spp., *Clonostachys rosea*, *Acremonium* spp. (*Neotyphodium*) and *Lecanicillium lecanii*. They were characterized as endophytes due to their isolation from asymptomatic plant tissues (Cherry et al., 1999; Cherry et al., 2004; Orole and Vega et al., 2008; Adejumo, 2009; Blackwell et al., 2009). Endophytic fungi are mostly host-specific, causing little or no risk to non-target organisms or beneficial insects, such as pollinators. This is an advantage, comparing with chemical pesticides, and can be considered as biological control of pests (Roberts and Humber, 1981; Smith et al., 1981). In addition, some endophytic fungi were re-isolated from colonized host plants, after artificial inoculation, including the following fungi: *M. anisopliae* (Fuller-Schaefer et al., 2005; Akello and Sikora, 2012; Greenfield et al., 2016), *B. bassiana* (Bing and Lewis, 1991, 1992; Wagner and Lewis, 2000; Parsa et al., 2013; Russo et al., 2015; Greenfield et al., 2016), *Fusarium oxysporum*, *Hypocrea lixii*, *Giberella moniliformis* and *Trichoderma asperellum* (Akello, 2012; Akello and Sikora, 2012; Akutse et al., 2013), showing their capability to colonize plants through an artificial inoculation and their potential to be used as biological control weapons. A complete review about the methods of artificial inoculation of endophytic fungi in plants can be found in (Bamisile et al., 2018a,b).

Endophytic fungi that are considered insect pathogens can directly suppress other plant pathogens through different mechanisms such as parasitism, competition for nutrition and ecological niches, and also due to the production of bioactive secondary metabolites (Jaber and Ownley, 2018). Virulent isolates usually express spore-bound proteases, in addition they produce and release hydrolytic enzymes, as mentioned before, during cuticular penetration, and also synthesize toxins (Vey et al., 2001; Freimoser et al., 2005; Shah et al., 2005; Qazi and Khachatourians, 2007; Zimmermann, 2007a,b; Khan et al., 2012a,b).

Therefore, the incorporation of endophytic fungi that have entomotoxic properties, as agents used in Programs of Integrated Pest Management (IPM) has been extremely effective and promising (Bamisile et al., 2018a,b). Understanding better the effect of ecological factors and inoculation methods on endophytic fungi virulence and colonization ability will help the users into developing an effective strategy for the control of target insect pests (Bamisile et al., 2018a,b).

25.4.2 Antifungal properties

In agriculture, phytopathogenic fungi can cause several diseases in plants; they are one of the most harmful phytopathogens, causing significant damages in most agriculturally important crops across the globe. Microorganisms as biological control agents as part of IPM against fungi has been suggested as the most sustainable long-term solution, comparing with the use of synthetic pesticides (Dean et al., 2012). The production of metabolites by these fungi is a strategy to guarantee their survival against pathogens. These fungi produce compounds that have antimicrobial activities; therefore, they ensure the protection of the plant against pests (Poling et al., 2008).

Mycoparasitism of the endophytes against pathogenic fungi involves the hydrolysis of fungal cell wall due to the production of enzymes that break down cell wall components (Askary et al., 1997; Zeilinger et al., 1999). Furthermore, the release of antifungal metabolites that permeate the injured hyphae and prevent a new synthesis of the host cell wall (Askary et al., 1997; Lorito et al., 1996) and growth of the host hyphal cytoplasm (Inbar et al., 1996). Species of the genus *Trichoderma* are potential biocontrol agents against several phytopathogenic fungi. One advantage for its effective use is an efficient coiling process followed by production of enzymes (Almeida et al., 2007). They are considered parasites of other fungi. Leastways some strains establish vigorous and long-last colonization of root surfaces and penetrate into the epidermis (Harman et al., 2004).

The endophytic fungus *Choiromyces aboriginum* MülW1C6, *Stachybotrys elegans* Mt2W1C1, and *Cylindrocarpon* sp. 5/97-12 and Mt2W3C4 were isolated from roots of common reed (*Phragmites australis*). These fungi were able to inhibit soil phytopathogenic fungi and produced β -1,3 glucanases and chitinases (Cao et al., 2009). *Chaetomium spirale* ND35 was isolated from *Populus tomentosa*. This endophytic fungus inhibited the growth of *Rhizoctonia solani* and caused morphological changes in the fungal cell wall. There were indications of the production of β -1,3 glucanases by *C. spirale* (Gao et al., 2005).

25.5 Conclusions

Filamentous fungi are microorganisms capable to produce a vast range of bioactive metabolites and are considered as excellent biofactories. Endophytic fungi, in particular, are important due to their interaction with plants, producing important metabolites and enzymes that help the plant in the defense against phytopathogens and maintenance of plant

homeostasis. The discovery of new active metabolites produced by endophytic fungi is challenging and can bring us substantial benefits in the development of transgenic plants, more resistant to pathogens, and also new drugs to combat human diseases.

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Chapter 26

Using molecular techniques applied to beneficial microorganisms as biotechnological tools for controlling agricultural plant pathogens and pest

Liliana Aguilar-Marcelino¹, Pedro Mendoza-de-Gives¹, Laith Khalil Tawfeeq Al-Ani^{2,3},
María Eugenia López-Arellano¹, Olga Gómez-Rodríguez⁴, Edgar Villar-Luna⁵ and
David Emmanuel Reyes-Guerrero¹

¹Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Mexico, ²Department of Plant Protection, College of Agriculture, University of Baghdad, Baghdad, Iraq, ³School of Biology Science, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia, ⁴Fitopatología, Colegio de Postgraduados, Texcoco, Mexico., ⁵CONACYT-Instituto Politécnico Nacional, CIIDIR-IPN, Unidad Michoacán, Jiquilpan, Mexico.

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26.1 Introduction

Biological control is an ecological and sustainable method to reduce parasitic populations to an acceptable level by conserving these populations at a non-detrimental level thanks to antagonistic organisms or natural living enemies. It is

fundamental to understand that biological control is a regulatory measure whose objective is not to eliminate the target organism, but to control its population to reduce its harmful effects. The soil is a habitat where different taxonomic groups of organisms share the same space, establishing different biological associations among them. The plant pathogens and pest have several natural enemies, among the main enemies of the nematodes in the soil are various groups of organisms including: protozoa, viruses, turbellaria, insects, mites, tardigrades, and nematode predators of other nematodes, bacteria and fungi. The use of new molecular and omic techniques opens up the possibility of identifying and understanding the mechanisms of action of the different natural enemies of plant pathogens and pest.

26.2 Diversity of plant pathogens

Soil microorganisms co-exist with organisms of different taxa and different biological associations are established among them (Rønn et al., 2012). Soil microorganisms help to plant hosts to modulate different plant processes such as, suppressing plant immunity (Upson et al., 2018). Plant pathogenic organisms include: viruses, bacteria, fungi, nematodes, mites, and insects that continuously affect economically important crops (Abdulkhair and Alghuthaymi, 2016). Some of these plant pathogenic microorganisms are briefly mentioned as follows:

26.2.1 Viruses

Viruses are small obligate intracellular parasites containing either RNA or DNA in their genome, which is protected by a virus-coded protein coat (Gelderblom, 2015). Viruses cause important diseases in a wide range of crops affecting agriculture production worldwide (Bernardo et al., 2018). Viruses can be transmitted from one plant to another plant by vegetative propagation, by mechanic transmission through the use of agricultural tools *i.e.*, through pruning scissors and also through pollen and seeds and mainly through insect vectors mainly insects (aphids, whitefly, trips, etc.). These vectors are capable to cause wounds in the plants that allow the horizontal spread of phytopathogenic viruses (Sepúlveda, 2011). Some viruses cause severe damage to economically important crops *i.e.*, the Tomato torrado virus (ToTV) cause burn-like symptoms on leaves, leaflets and upper stem parts in tomato and can affect the whole plant and leads to death (Wieczorek et al., 2019). Other viruses affecting different host crops are transmitted by different vectors; for instance, the Banana bract mosaic virus (Potyvirus) is transmitted by an aphid; the Citrus leprosis virus (Cilevirus) is transmitted by a the Citrus yellow mosaic virus (Badnavirus); The Mayze fine streak virus (Nucleorhabdovirus) is transmitted by a leafhopper; The sorghum mosaic virus (Potyvirus) transmitted by an aphid; Papaya leaf distortion mosaic virus (Potyvirus) is also transmitted by an aphid, the Rice necrosis mosaic virus (Bymovirus) it is transmitted by fungus (Geering and Randles, 2012). These are only some examples of viruses affecting different crops; however, soil harbor many novel viral species representing a large genetic reservoir (Johns, 2017). Understanding the role of viruses in the soil ecosystem can lead to establish strategies of control of viral diseases that affect crop production worldwide.

26.2.2 Bacteria

Plant pathogenic bacteria (PPB) are important plant pathogens widely spread all over the world (Bar-On et al., 2018). It is estimated that from 7100 classified bacteria about 150 species are responsible for different plant diseases (Rajesh-Kannan et al., 2016). PPB has been classified into three families: Xantomonadaceae, Pseudomonaceae, and Enterobacteriaceae. These bacterial families harbor the following genera: *Dickeya*, *Liberibacter*, *Erwinia*, *Pectobacterium*, *Candidatus*, *Pantoea*, *Agrobacterium*, *Pseudomonas*, *Ralsonia*, *Burkholderia*, *Acidovorax*, *Xanthomonas*, *Clavibacter*, *Streptomyces*, *Xylella*, *Spiroplasma*, *Phytoplasma*, *Brenneria*, *Lonsdale* and *Xylophilus* (Agrios, 2005). PPV infect plants through different mechanisms; for instance, through the use of cell wall degrading enzymes (cellulases, xylanases, pectinases) or by injecting chemicals *i.e.*, *hrp* harpins, and *Avr* proteins associated with plant diseases (Alfano and Collmer, 1997). The main symptoms in the crops include abnormal growths on roots, stems, spots on leaves or fruit, blights or deadening of tissue on leaves, stems or tree trunks, and rots of any part of the plant, usually roots or tubers (Vidaver and Lambrecht, 2004).

26.2.3 Fungi

The kingdom fungi represent one of the widest phyla of organisms spread all over the world inhabiting all substrates and climate conditions (CK-12 Foundation, 2019). It is estimated that at least 18,000 species of fungi have been described (Marin-Felix et al., 2017). Fungi contain five true phyla including Chytridiomycota, the Zygomycota, the Ascomycota, the Basidiomycota, and Glomeromycota. The Deuteromycota is an informal group of unrelated fungi that

all share a common character – they use strictly asexual reproduction (ERS, 2019). Some of the fungi species are being identified as responsible of most economically important plant diseases and production many dangerous mycotoxins (Attitalla 2010a,b). For instance, *Magnaporthe oryzae*, causes the Rice blast disease (Khush, 2005), *Botrytis cinerea*, causes the gray mold in tomato plants (Ongouya-Mouekouba et al., 2013), *Puccinia* spp., causes black rust of cereals (SENASICA, 2016), *Fusarium graminearum*, reduces the quality of grains and contaminate grains with mycotoxins of impact in public and animal health (Agris, 2005; Michielse and Rep, 2009); meanwhile, *Colletotrichum* spp. causes Anthracnose spots and blights of aerial plants and post-harvesting infection (Prusky, 1996), *Ustilago maydis* causes tumor-type galls, that actively grow in corn tissues (Brefort et al., 2009). *Melampsora lini* produces soybean and coffee disease called “Rolla” (Hull, 2002). Fungal plant diseases are complex diseases that require an integrated control program to attack fungi from different control measures.

26.2.4 Nematodes

Nematodes occupy an important place in soil; due to their abundance. It has been estimated a nematode density of 20×10^6 nematodes approximately per square meter of soil (Barron, 1977). Some groups of nematodes are beneficial; since act as decomposers of organic matter, cycling nutrients and fertilizing the soil (Johns, 2017); although, there is a number of genera/species causing important diseases in economic important vegetables and crops (Bernard et al., 2017). For instance, the root-knot nematodes i.e., *Meloidogyne* spp. are parasites of tomato and other economically important vegetables; they feed on the root tissues, causing root galls; other nematodes penetrate the roots and after female dies her body containing live eggs (cysts) can remain for many years as a strategy of survival in nature; other kinds of nematodes feed on plant foliage or cause burrowing and lesions in the roots; likewise, there also exist foliar and reniform nematodes (Schmitt and Sipes, 2014). Beyond that, some nematodes can also act as virus vectors (Mitiku, 2018). Other important pathogenic nematodes include: the cyst nematodes i.e., *Heterodera* and *Globodera* spp.; the root lesion nematodes i.e., *Pratylenchus* spp.; the burrowing nematodes *Radopholus similis* and *Ditylenchus dipsaci*; the pine wilt nematode *Bursaphelenchus xylophilus*; the reniform nematode *Rotylenchulus reniformis*; *Xiphinema index*; *Nacobbus aberrans*; and *Aphelenchoides besseyi* (Jones et al., 2013). The severe economic consequences of phytonematodes to crop production is a concerning problem that encourages scientists to find sustainable and effective methods of control.

26.2.5 Insects and mites

Insects occupy one of the widest varieties of genera/species living in all types of environments (Navarajan, 2007). It is estimated that insects affect 18% of world agricultural production (Jankielsohn, 2018). The plant pathogenic insects and mites can be classified according to their target plant; for instance, insect pests of cereals and grains, pests of pulses, pests of Tuber crops, pests of Citrus crops, pests of cole crops, insect pests of fruit trees, pests of temperate fruits, etc. These plague insects include hoppers, flies, aphids, sawflies, beetles, mites, caterpillars, ants and others (Ruiz-Corral et al., 2013). Some of the most important insects affecting crops include: the shoot and fruit borer *Leucinodes orbonalis* (Lepidoptera) affects brinjal; the spotted leaf beetle *Henosepilachna vigintioctopunctata* (coleoptera) affects brinjal and other plants, the gray weevil *Mylocherus subfasciatus* (Coleoptera) affects cotton, sorghum, pear millet and maize (Navarajan, 2007); likewise, the stem borer caterpillar affects rice, sorghum, finger-millet, maize, pearl-millet, sugarcane, mango, fig, rubber, jackfruit, eucalyptus, mulberry; the Pod sucking bugs affect pulses, safflower, chilli pepper, sorghum, groundnut, tomato, cotton; Rootborer affect corn and sugarcane; weevils affect Coconut, jute, cotton, sorghum, pearl millet, maize; the Mealybugs affect beans, cashew, cassava, coffee, cocoa, citrus, cotton, groundnut, guava, jute, sugarcane, sweet potato, tomato, apple, apricot; the Berry borer affects coffee; the fruit and shoot borer affect brinjal, potato, tomato, maize, sorghum, cotton, sunflower, pigeonpea, chickpea, field beans, roses; the Aphids affect Wheat, barley, oats, ladyfinger, brinjal, guava, chilli pepper (The Economic Times, 2013).

26.3 Conventional methods of control of agricultural plant pathogens and pests

The management of the crop-pests-environment system follows the principles of pest control that correspond to exclusion, eradication, and protection. What is required in the first instance is exclusion, that is, to put a barrier to prevent the entry of a pest in a place where it is not present through a legal framework between countries and within each country. In Mexico, the potato tuber nematode *Ditylenchus destructor* is of quarantine significance so there is a legal framework related to the internal and external regulation of the mobility of plant products susceptible to this nematode with the purpose of containing or confining its presence in Mexico, for which the official Mexican Norms NOM-007-FITO-

1995, NOM-009-FITO-1995, NOM-012-FITO-1996 as well as the regional norms represented by the North American Plant Protection Organization (NAPPO) were issued. Made up of Canada, United States, and Mexico are the Regional Standard for Phytosanitary Measures RSPM No. 3 (SENASICA, 2013). On the other hand, once a pest is established in a new agricultural region, its eradication proceeds in order to avoid its establishment, otherwise the protection of the crop is carried out with the use of different control methods whose purpose is to lower the level of the pest in such a way that the yields are economically profitable.

26.3.1 Conventional methods

The conventional methods of control of chemical, physical, biological and cultural pests are used in an integrated way in whose focus is the management of the crop and vary from one place to another so it is essential to evaluate its feasibility in each region. A characteristic of technified agriculture is the dependence on the use of agrochemicals to avoid losses due to the attack of pests. Currently, the trend is towards lower consumption of agrochemicals, for example, in the Valley of Culiacán, Sinaloa, Mexico, an active ingredient consumption of 54.5 kg/ha was reported during the 1997–98 cycle and 0.263 kg/ha during the year 2012 (Leyva Morales et al., 2014), likewise, the use of new compounds such as verango® is reported for the control of the *Meloidogyne incognita* root-knot nematode in tomato production (*Solanum lycopersicum*) (Carrillo-Fasio et al., 2016). On the other hand, the biological control is complementing the chemical control thus it has that of the insecticides used in Mexico, 6.2% corresponds to the biological type where it stands out the use of the following species in the cultivation of vegetables: *Bacillus thuringiensis*, *Trichogramma* sp., *Phaeoacremonium foveolatum* and *Bauveria bassiana* (Pérez-Olvera et al., 2011). In relation to cultural control corresponds to all those cultivation practices that favor the crop and lower the level of the pest, such as crop rotation, planting dates, sanitation creating unfavorable conditions, host eradication, planting spacing, mulches, pruning, fertilization, incorporation of waste, intercropping, mainly. The intercropping has been a common practice in traditional agriculture in Mexico, particularly the intercropped system corn-beans and corn-squash. The intercropping with marigold on tomato induced a reduction in tomato early blight caused by the fungus *Alternaria solani*, this by an allelopathic effect of marigold on *A. solani* conidia germination, by altering the microclimatic conditions around the canopy, particularly by reducing the number of hours per day with relative humidity $\geq 92\%$, thus diminishing conidial development, and by provide a physical barrier against conidia spreading (Gómez-Rodríguez et al., 2003), in the same way, the intercropping with marigold on tomato showed a reduction in the infection of tomato roots by *Nacobbus aberrans* at the end of the crop (Zavaleta-Mejía and Gómez, 1995). The use of resistant varieties contributes to long-term pest management, so it is necessary to identify resistant genotypes and introduce, by breeding, the resistance genes in commercial varieties (Richins et al., 2010). The Serrano pepper Criollo de Morelos CM-334 native of Mexico, it has shown resistance against all isolates of *P. capsici*, which is why it is considered as universally resistant (Glosier et al., 2008; Lamour et al., 2012), in the same way CM-334 presents resistance to the three major *Meloidogyne* species [*M. incognita*, *M. arenaria*, and *M. javanica*] (Pegard et al., 2005), unfortunately, CM-334 is susceptible to *N. aberrans*. Within the search for resistant genotypes are reported Serrano pepper lines 41-1 and 41-2, and Huacle pepper line 35-3 from the region of Puebla and Oaxaca, Mexico with resistance to all three pathogens evaluated *P. capsici* and nematodes *Meloidogyne incognita* as *N. aberrans* (Gómez-Rodríguez et al., 2017). It is important to take into account that the sum of different phytosanitary and crop management practices favors the sustainability of agricultural production.

26.4 Biological control of agricultural plant pathogens and pests

The challenges for the new millennium proposed by the United Nations included stopping hunger, achieving the food security and better nutrition through promoting sustainable agriculture (FAO, 2017). These approaches need to consider that global agriculture faces a number of problems derived from the high costs of fertilizers and chemical inputs (Crop Production Costs, 2019), the dependence of agrochemical to increase the production (Gill and Garg, 2014) as well as a number of severe diseases caused by a diversity of reported and non-reported pests to the main crops (Bebber et al., 2019). Traditionally the method of control of plague affecting crops, all over the world is the use of chemical pesticides that help to reduce the damage in crops; however, during the last decade's plagues have developed resistance against most of the commercially available chemical molecules that dramatically decreased their effectiveness (IRAC, 2019). On the other hand, there exist other important limitations in the use of this method that is the misuse and poor handling of chemical pesticides that are hazardous to the health of farmers, consumers and to the environment (Okonya et al., 2019). The new tendencies for agricultural production are focused on obtaining safe and

contaminant-free food (Madhusudhan, 2016). In this context, searching for alternative and sustainable methods of control is an increasing worldwide demand.

26.5 Methods of control of plague of importance in agriculture

The enormous losses in agriculture occasioned by pests and the undesirable consequences in the use of chemical pesticides have motivated an intense searching of strategies sustainable control different from the use of pesticides. The establishment of alternative methods of control of agricultural pests has to be accompanied by important recommendations considering cultural practices, variety selection, biological control and restricted use of pesticides only used on an-needed basis (Stewart and McClure, 2019). Some of the most important strategies of control of agricultural pests are briefly discussed as follows: Cultivar breads (Noureldin et al., 2013); Crop rotation (Spencer, 2018); Intercropping (Bybee-Finley and Ryan, 2018); Undersowing (Känkänen, 2010); Natural products (Al-Ani et al., 2012; Mohammed et al., 2012; Egbuna et al., 2019; Beck et al., 2018) and Biocidal plant extract (D'Addabbo et al., 2014). In addition to all these control measures, an eco-friendly method of control based in biological associations in nature has been proposed to reduce the population of other species regularly a pathogen or undesirable as biological control. Biological inter-relationships among organisms in nature include commensalism, mutualism, parasitism, neutralism, competition, and predation (Leun and Poulin, 2008).

26.6 Biological control

Biological control is an alternative method of control that aims at reducing the population of pest species by favouring indigenous natural enemies, or by introducing new ones (Delattre et al., 2019). Before establishing any method of control it is important to consider that an organism designed as a suitable biological control agent should fill some minimum requirements as follows: (1) biological control is less costly and cheaper; (2) natural enemies give protection to the crop throughout the crop period; (3) biocontrol agents do not cause toxicity to the plants or crops; (4) application of biocontrol agents (natural enemies) is safer to the environment and to the person who applies them; (5) they multiply easily in the soil and leave no residual problem; (6) biocontrol agents not only control the disease but also enhance the root and plant growth by way of encouraging the beneficial soil microflora. It increases the crop yield also; (7) biocontrol agents are very easy to handle and apply to the target; (8) Biocontrol agent can be combined with bio-fertilizers; (9); biological control agents are easy to manufacture; (10) it is harmless to human beings and animals (Sharma et al., 2013). When a method of biological control is intended to be established it is important to consider different aspects related with ecology, physiology, environment, microhabitat climate, and especially in the life cycle of both organisms that will biologically interrelate (Heimpel, 2017). It is also important to consider that biological control organisms should be specifically blanked to a plague blank and it should not affect other microorganisms in the ecosystem (Thurman et al., 2017). In the present chapter, a general view of the most representative biological control agents of different plant-pathogen is briefly revised.

26.6.1 Biological control of insects affecting crops

Potential agents of biological control of plant-pathogen insects are being identified and these organisms include bacteria, fungi, mites and a number of predator insects *i.e.*, lady beetles, ground beetles, rove beetles, flower bugs and other predatory true bugs, lacewings and hoverflies (Stone, 2019) catch and eat their prey. Some common predatory arthropods include ladybird beetles, carabid (ground) beetles, staphylinid (rove) beetles, syrphid (hover) flies, lacewings, minute pirate bugs, nabid bugs, big-eyed bugs, and spiders (Evans et al., 2011).

26.6.2 Entomopathogenic bacteria

The potential use of natural antagonists of insects was proposed since centuries ago and it took a big boom at early 30s (Seetman and Howard, 1936). Since that time a number of biological systems of control have successfully helped to diminish the havoc caused by insects, nematodes and other important crop plagues. Some of the most widely studied natural antagonists of pests affecting plants include some Bacteria *i.e.*, *Bacillus thuringiensis*; this is a gram (−) bacterium living in soil that grows through vegetative cells harboring spore and also some small crystals of protein nature that have been involved in the death of a number of insect or nematode plague (Sauka and Benintende, 2008). These crystals also called “Cry proteins” are highly specific for structures situated on the intestine cells of the insects. Once

plague insects consume specific *Cry* proteins and interact with some specific receptors presents in intestinal cells of the blank insect, an enzymatic and mechanical process take place and a porous in the intestinal cell is produced provoking an unbalance in the epithelial cellular architecture with loss of the capability of nutrient absorption followed by the death of the insect (Endo et al., 2018). The great advantage in the use of *B. thuringiensis* for controlling pests of importance in agriculture is precisely its high specificity for particular target plague (Endo et al., 2018). Other bacteria attacking plant-pathogenic insects include enterobacteria *i.e.*, *Enterobacter* sp. affecting the fruitflies *Ceratitis capitata* and *Anastrepha fraterculus*, causing septicemia, feeding inhibition, lack of motility and death (Mampallil et al., 2017); and also *Clostridium bifermentus*, affecting mosquitos and flackflies (Nicolas et al., 1990).

26.6.3 Entomopathogenic fungi

Entomopathogenic fungi are a group of fungi living in soil that infect insects by penetrating their cuticle to penetrate their bodies to eventually kill them and feeding on them (Dara, 2017). Entomopathogenic fungi that are classified into the divisions Ascomycota, Zygomycota, Deuteromycota, Oomycota, Chytridiomycota (Esparza-Mora et al., 2017). These fungal pathogens are being identified as important contributors to insect population dynamics in soil. Derived from many years of study and about 800 fungi species identified as pathogenic for insects many commercial mycoinsecticides are being developed; however most of these products are based in only three genera/species of entomopathogenic fungi: *Beauveria bassiana*, *Metharhizum anisopliae* and *Isaria fumosoroseus* (Maina et al., 2018). Entomopathogenic fungi produce conidia that attach to the insect cuticle and an enzymatic production process mainly through fungal protease production takes place; these enzymes together with a mechanical process through appressoria development brake the insect cuticle and penetrate the body insect to invade and kill the insect taking their nutrients from the insect tissues (Brunner-Mendoza et al., 2019).

26.6.4 Entomopathogenic nematodes

Entomopathogenic nematodes are considered some of the main insect antagonists in nature that is a very useful biological tool of control of important insect plague in many countries around the world (Marianelli et al., 2017). The most widely studied entomopathogenic nematodes belong to the families Steinernematidae and Heterorhabditidae. They are free-living organisms that act as predators of insects; they possess symbiotic/mutualistic bacteria that help to destroy and degrade their prey insect of the wide diversity of plant-pathogenic insect hosts and they are considered as potential biological control agents of important insect plague (Lima de Brida et al., 2017). Entomopathogenic nematodes are obligate parasites of insects and they have important biological strategies against their prey nematodes; for instance, they are capable to produce insect suppressing host immunity (Lu et al., 2017). Excellent results about the use of entomopathogenic nematodes *i.e.*, *Steinernema* spp. against different insect pests including Coleoptera (Gangadhara et al., 2019); Lepidoptera (Cruz-Martínez et al., 2017); Hemiptera (Batista and Machado, 2010) and Diptera (Kamali et al., 2013).

26.7 Biological control of nematodes affecting crops

26.7.1 Bacteria

The most widely studied bacterium that acts as a natural nematode enemy and it has used as a potential biological control agent is *Bacillus thuringiensis*. *B. thuringiensis* is a gram (+) bacterium that produces crystals of protein nature. These crystals are being involved in the control of a number of diverse plagues affecting cops including nematodes (Wang et al., 2012; Fortea et al., 2017). The mechanism of infection of *B. thuringiensis* against nematodes is similar to the one happening with insect pests. The nematode consume the crystals, it is a process of recognition with the membrane receptor and an enzymatic process mediated by proteases take place and intestinal cells are destroyed and the absorption of nutrient is lost followed by inanition and death of nematode. The lethal effect of *B. thuringiensis* against nematodes it seems to be due to the production of α endo-toxins by the bacterium (Mohamed et al., 2008). The nematophagous bacterium *Bacillus nematocida* is an aerobic gram (+) bacterium spore forming that possess an ability to kill nematodes (Huang et al., 2005). This bacterium infects nematodes using a 'Trojan-horse' -like mechanism (Niu et al., 2007). Bacteria are ingested by nematodes; once bacteria are swallowed by nematodes, they colonize in the intestinal tract of nematodes and secrete virulence factors in the form of proteases to cause nematode death (Lian et al., 2006). Other bacteria have been explored as potential agents of control of plant-parasitic nematodes. For instance, *Pasteuria*

penetrans. This bacterium is a gram (+) bacterium living in soil that produces spores that attach to the nematode cuticle and also through an enzymatic and mechanic mechanism break the cuticular wall and penetrate the nematode integument to produce a large number of microcolonies into the nematode body causing several damages to nematodes. This biological system has been deeply studied and demonstrated its successful against root-knot nematodes affecting tomato plants and other economically important crops (Kumari, 2017).

26.7.2 Nematophagous fungi

Nematophagous fungi are soil micro-fungi that are natural-nematode enemies. These fungi form part of the normal soil microbiota and there exist three groups of nematophagous fungi in relation to their mode of action and nematode stage target as follows: the nematode-trapping fungi, the endoparasitic or endozoic fungi, and the egg- and cyst-parasitic fungi (Nordbring-Hertz et al., 2006).

26.7.3 Nematode -trapping fungi

Nematode trapping fungi are saprophytic microorganisms in soil; however, when they are exposed to nematodes they are able to transform their mycelia entrapping devices specially designed for trapping nematodes becoming nematode-predatory facultative fungi (Mendoza de Gives and Torres-Acosta, 2012). Some of the most studied genera/species of nematophagous fungi found under different geographical areas all over the world include *Arthrobotrys* spp., *Duddingtonia flagrans*, *Monacrosporium* spp. and *Dactylaria* spp. (Dasgupta and Khan, 2015). Nematode-trapping fungi have demonstrated to be promising biological control agents against phytonematodes affecting economically important crops. Some examples of successful assessment in the use of nematode-trapping fungi in the control of different phytoparasitic nematodes in different crops include the following ones: *Arythrobotrys oligospora*, *Candelabrella musiformis*, *Dactylaria eudermata* against *M. incognita* in Tobacco plants (Hastuti and Faull, 2018); *Meria coniospora* against Root-knot nematodes in tomato plants (Jansson et al., 1958); *Arthrobotrys oligospora* against *M. incognita* in tomato plants (Singh et al., 2012).

26.7.4 Nematophagous fungi parasites of nematode eggs

Other groups of nematophagous fungi is those that produce hyphae invading phytoparasitic nematode eggs becoming truly ovicidal fungi (Mendoza de Gives and Ribeiro-Braga, 2017). These fungi are able to develop mycelia that have affinity to grow on the nematode egg-shell and form structures called appressoria that form an infective tube which fungus uses to penetrate through an enzymatic process where the egg-shell is degraded and penetrated by hyphae to colonize the egg to eventually destroying and feed on the embryo inside the egg (Devi, 2018). Some cases of successful assessment in the use of ovicidal fungi in the control of phytoparasitic nematode eggs include *Pochonia chlamydosporia* and *Purpureocillium lilacinum* against *M. incognita* (Devi, 2018); *Paecilomyces lilacinus* against *Meloidogyne* spp. in tomato plants (Siddiqui et al., 2000). The benefits of the biological control of pest affecting economically important crops are priceless and not only improving the crop productivity and in terms of healthy food but the benefit involves to the environment since an eco-friendly system of production. The use of biological control measures is crucial mainly for organic farming all over the world.

26.8 Molecular techniques applied to the identification of candidate microorganisms for biocontrol

Through the last decades, molecular tools have contributed to the diverse plant pathology and pests of the crop in order to look for other strategies of control. Specifically, plant pests have been evolved diverse mechanisms to invade and outwith their hosts, and these offer opportunities for the development of biocontrol measures. For instance, there is a great concern about environmental damage and residues in crops following treatments against plant pathology and pests of the crop. Molecular tools have been increasingly used in the identification of different taxa, biology, trophic chains and diagnosis of the most important pests that cause severe damage to multiple crops. In this regard, the polymerase chain reaction (PCR) is one of the main tools used for the molecular identification of organisms as fungi, bacteria, virus and nematodes, several of them beneficial (biological control agents) or harmful (plant pathogens) in agricultural production systems. The PCR allows the specific and exponential amplification of a predetermined DNA region by the use of two small, specifically designed fragments of DNA (primers or oligonucleotides), which form the two termini of the

nucleic acid molecule to be amplified (van Pelt-Verkuil et al., 2008). There are several variants of this technique that are currently used in molecular diagnostic laboratories, for example, RACE-PCR (Rapid amplification of cDNA ends), RT-PCR (Reverse transcription polymerase chain reaction), gene cloning, nested-PCR and qPCR (real-time PCR) (Narayanasamy, 2011). The PCR technique modified the traditional strategies of study in the worldwide. In addition, the PCR has adopted distinctive assays in omics areas, including proteomic, genomic, transcriptomics tools of crop studies, this technique calls New Generation such as Massive Sequence. Specific studies have been carried out to identify suitable targets for the developing of pests' resistance crops. The microbial community in the rhizosphere harbors organisms like viruses and viroids, bacteria, fungi, and nematodes, several of them beneficial (biological control agents) or harmful (plant pathogens) in agricultural production systems. Microorganisms that adversely affect plant growth and health are the pathogenic viruses and viroids, bacteria, fungi, oomycetes, and nematodes, whereas microorganisms that are beneficial include nitrogen-fixing bacteria, endo- and ectomycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) and fungi (Raaijmakers et al., 2009). On the other hand, plants can also interact with many species of insects during their lifetime. These insects include harmful herbivores above and below ground, as well as beneficial natural enemies of herbivores and pollinators (Poelman and Dicke, 2014).

26.8.1 Bacteria

The identity of bacterial plant pathogens is essential to strategic and sustainable disease management in agricultural systems. It is important to know the identity of bacterial populations that are useful in agriculture because of the benefits they have on the growth and development of plants. Molecular markers such as 16S, 23S rRNA sequences or intergenic sequences are useful for distinguishing microorganisms among various genera, species and even to differentiate among strains. By using the PCR-based techniques the detection, identification and quantification of microorganisms can be performed and complemented with culture-dependent and biochemical methods. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500 bp) is large enough for informatics purposes. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rRNA gene sequencing studies as opposed to the more cumbersome manipulations involving DNA-DNA hybridization investigations (Janda and Abbott, 2007).

26.8.2 Fungi

Several studies related to the identification of these organisms have been documented. A 27% of the studies used molecular data only (mostly from the internal transcribed spacer (ITS) region) for fungal identification; and ~14% used a combination of morphology and molecular data (both rRNA and protein coding genes) to identify fungi (Raja et al., 2017). In early identification studies of fungi, both the mitochondrial rDNA and the Internal Transcribed Spacer (ITS) region, Small Subunit (SSU), and Large Subunit (LSU) of the nuclear rDNA were examined. Synthesis of universal and fungus-specific primers enabled a great leap forward in the identification of fungal communities. The ITS1-5, ITS1F, and ITS4B primers developed in these seminal studies are still widely used, although several alternative and improved modifications are available for nearly all of these (Tedersoo and Nilsson, 2016). A review of this topic has been widely discussed by Gherbawy and Voigt (2010). Thus, the incorporation of molecular techniques into what has been traditionally considered as morphology-based taxonomy of fungi helps us in the differentiation of fungal species and varieties. Databases of genomes and genetic markers used as sources for molecular barcodes are being created and the fungal world is in progress to be unveiled with the help of bioinformatics tools. Genome projects provide evidence for ancient insertion elements, proviral or prophage remnants, and many other patches of unusual composition. DNA sequences and other genetic markers provide large amounts of data which are cultivation-independent and do not depend on physiological inconsistencies. Genetic markers constantly reflect the identification treasure hidden in the genetic information and allow to control the degree of resolution by choosing the appropriate genes.

26.8.3 Nematodes

Different microorganisms are frequently polluting crops affecting farmers' economy. Plant parasitic nematodes are classified into four main groups: (a) Migratory ectoparasites, which remain outside the root and feed by using the stylet to

pierce epidermal cells and/or cells deeply localized inside the root. (b) Sedentary ectoparasites, whose habitat is outside of the root, choose a single site or cell to feed off a prolonged period of time in order to obtain nutrients. (c) Migratory endoparasites are one of the most destructive parasitic nematodes because they can cause extensive damage during penetration and migration and while feeding in the plant and (d) Sedentary endoparasites are a group of nematodes, which have developed specialized feeding sites to survive in their hosts for long periods without causing the plant death (Whitehead, 1998). Based on the knowledge of these pathogens strategies of control are available in the market, one of them is the use of *Bacillus* spp. an entomopathogenic gram (+) bacteria (De Maagd et al., 2003). For instance, *B. thuringiensis* (*Bt*) is a well-characterized entomopathogenic which proteins have been purified in order to classify them in different clades. The *Bt* studies have required the used of chromatography and SDS-PAGE to support its identification and protein confirmation through cloning, end-point PCR, genetic engineering and bioinformatics. Different *Bt* strains are nowadays used as commercial biocontrol agents with the advantage to protect different crops and the environment from pesticides pollution. Another research area using *Bt* is the evaluation of specific Cry and Cyt proteins that target phytonematodes (*M. incognita* and *Radopholus similis*) (Mohamed et al., 2008) and free-living nematodes (*Panagrellus redivivus*, *Caenorhabditis elegans*) (Wei et al., 2002; Niu et al., 2007).

26.8.4 Insects and crop domestication

Most emerging infectious diseases today are arthropod-borne that cannot be easily controlled. Because the use of molecular techniques is rapidly growing as tools, treatments with biological methods have become more diverse and powerful and easy to implement. In addition, the big diversity of antagonists against crop-pathogens are identified to improve their control with genetic technics such as RAPD/ISSRs/UP-PCR and AFLPs considering the most pertained tools for identification of different antagonist strains closely related (e.g. *Bacillus* spp. strains) that have been used towards insect belonging to the orders of Lepidoptera, Diptera and Homoptera, showing a wide spectrum of control through the main Cry proteins (DeMaagd et al., 2003). A similar method of identification has been carried out with *Trichoderma* and *Trichogramma* species against different mycotoxins (Kullning et al., 2000; Santos et al., 2015). Crop domestication has been studied for more than a century and benefited recently technological innovations in genomics. Comparative analysis (whole sequence) of population genomics data of *Trichoderma* spp. and *Bacillus* spp. were studied from current samples with their old past bacteria as a wild variety (Herrera-Estrella, 2014; He et al., 2011). These kinds of antagonists have the benefit to enhance the resistance of plant against pathogens and also they are able to produce diverse enzymes to elicit a response in infected plants against insects. Also, fungi such as *Trichoderma* can promote the plant growth and carried the tolerance from abiotic stress (Saldajeno et al., 2014; Singh et al., 2014). The importance of other entomopathogenic fungi denominated *Metarhizium* spp. against multiple insect pests was study of the DNA sequence which has improved the information knowledge of *Metarhizium* species as Phylum against crop-pests like spittlebug (Hemiptera: Cercopidae) and locust (Orthoptera) that damage corn, sugarcane, and bean crops (Brunner-Mendoza et al., 2019). The authors have mentioned that with this technology the species has now been classified into 30 new species. In addition, each species of *Metarhizium* fungi appeared to show specificity and wide range against crop-insects. The biological control methods are sustainable systems against plant pathogens and pest, actually exist commercial formulations to reduce the negative effect of pesticides on environment and animal and human health. Biological products derivate from *B. thuringiensis* (spores and crystals proteins), and the mycoinsecticides such as *Trichoderma*, *Metarhizium*, *Beauveria bassiana*, *Isaria fumososea*, etc., are available in the market in benefit to farmers.

26.9 Omics: proteomics, metabolomics, peptidomics, transcriptomics, phenomics, interactomics, genomics

Many beneficial microbes are utilizing in controlling plant pathogens in the worldwide. These beneficial microbes have several mechanisms very effective against plant pathogens such as (A) parasitism, (B) producing the enzymes and secondary compounds, (C) Inducing the defense and resistance of the plant (Al-Ani, 2006; Al-Ani and Salleh 2010; Al-Ani and Al-Ani 2011; Mohammed et al., 2011, 2013, 2014; Al-Ani et al., 2013a,b; Al-Ani, 2017a,b, Al-Ani and Albaayit, 2018a,b; Al-Ani et al., 2018; Al-Ani, 2019a,b,c,d,e,f). The usage of some techniques to detect the mode of action for these mechanisms was limited. The techniques of omics comprise proteomics, metabolomics, peptidomics, transcriptomics, phenomics, interactomics, genomics, and secretomic (Sharma et al., 2017). The omics are showing very efficacy in detection the metabolome and expressome for several biocontrol agents such as *Trichoderma*. Lorito et al. (2010)

indicated the importance of omics in studying the complex and diverse biotic interaction for biocontrol agent such as *Trichoderma*.

26.9.1 Proteomics

The proteomic is more useful to study the interaction between the host and biological control agent against a pathogen, as well as, the interaction between a pathogen and biological control agents (BCAs). It can use the proteomic in the detection of many novel proteins that may extract and utilize in control of plant pathogens as an alternative to the chemical pesticides. The proteins of fungal cell wall-degrading enzymes (CWDEs) comprising three novel proteins with *N*-acetyl-b-D-glucosaminidase, and endochitinase that produced by *T. atroviride* as a response to plant pathogen *Rhizoctonia solani* (Grinyer et al., 2005). The plant pathogen *Botrytis cinerea* interaction with *T. harzianum* was induced production several CWDEs including β -1,3-glucanases, proteases, b-1,6-glucanases, chitinases, and xylanases in media (Yang et al., 2009). The CWDEs are detecting in media during the interaction between biocontrol agents such as *Trichoderma* and plant fungal pathogens leading to inhibit the growth of the mycelia. Interestingly, 230 extracellular proteins such as cellulases, lignin-degrading enzymes, hemicellulases, protein-translocating transporter, proteases, and other hypothetical proteins in the special substrate of *T. reesei* (Adav et al., 2012). In addition, the proteomic can be a help in detect the induced of plant immunity in plant treated with beneficial microbes such as *Trichoderma*. They detected many positive and negative proteins effectors in the interaction between maize root and *T. virens* that included multiple effectors for suppressing the defense pathways in the inducing systemic resistance (Lamdan et al., 2015).

26.9.2 Metabolomics

This analysis involves study the metabolites of BCAs and changes in the host tissues getting because of the interaction with BCAs. Metabolomics is helpful for detecting many secondary metabolites of *Trichoderma* including toxic metabolites for the plant, useful metabolites antagonistic against harmful microbes, and other metabolites to induce the defense and resistance in plant (Mukherjee et al., 2012). Mazzei et al. (2016) detected some changes in the tomato leaves after treating with secondary metabolites such as (1) harzianic acid and (2) 6-pentyl-2H-pyran-2-one of *Trichoderma*. They determined the metabolites activity of *T. harzianum* strain OMG-08 is involving to enhance the plant growth by measuring the changes of metabolites in the plant leaves (Vinci et al., 2018). The metabolomics is also used to determine the active metabolic pathways through the conidia germination of *T. atroviride*, which detected occurrence the increase in the main metabolite such as osmolytes, betaine, and mannitol (Kaliňák et al., 2014).

26.9.3 Peptidomics

Trichoderma is a resource of peptaibols. Schirmbock et al. (1994) found *T. harzianum* could inhibit the mycelium growth the plant pathogen *Botrytis cinerea* by producing the peptaibols trichorzianines A (1) and B-1. The two *Trichoderma* species such as *T. koningiopsis* and *T. gamsii* were able to produce 30 peptaibol sequences showing the inhibition effect against the filamentous fungi (Marik et al., 2018).

26.9.4 Transcriptomics

The transcriptomics is an interesting analysis for detecting whole transcriptional of RNA that useful for demonstrated the biosynthetic pathway of protein and enzymes for BCAs and the interaction between plant and BCAs. This analysis was provides a chance to get information about the trichodermin biosynthesis of *T. brevicompactum* (Shentu et al., 2014). They revealed the gene expression in outer root layer through colonization with *T. virens* that detected different genes encode comprising proteins, enzymes, and transporters (Morán-Diez et al., 2015). Transcriptomic study revealed many expression genes including 888 genes that called differentially expressed genes (DEGs) (Sun et al., 2016). The major role of DEGs connected with different processes that useful for encoding the secondary metabolite, enzymes, proteins, or transcription factors (Sun et al., 2016).

26.9.5 Phenomics

The measurement of phenomics was meaning the changes determination that possibly happens as a response to external (Environmental) and internal (Genes) conditions. This phenomics is very important to identify the characteristics of

BCAs. The identified several phenotypic of *Trichoderma* species that including the growth rate and colony morphology in different temperatures for the anamorph and teleomorph (Chaverri and Samuels, 2004). The phenomics is providing a chance to estimate the traits of BCAs such as *Trichoderma* species that can separate among the high number of *Trichoderma* isolates and taxonomy.

26.9.6 Interactomics

The biological study of interactions is between proteins inside cells and other interactions with molecules of cells for BCAs. The interaction between proteins and genes of BCAs are including the functional in the same pathway that leading to a particular phenotype. This interaction is affecting the efficacy of *Trichoderma* to antagonistic against plant pathogens. However, as for an example, the 14-3-3 proteins (a eukaryotic-specific protein family) played an important role in many signaling pathways in a plant that was interaction with defense-related proteins and be a target of pathogen effectors (Lozano-Durán and Robatzek, 2015).

26.9.7 Genomics

The genomic is involving the different study on genomes. The cluster the ancestral gene within AT-rich blocks of *Trichoderma reesei* (Li et al., 2017). The genomic analysis can utilize to determine the genome traits of mycoparasitic isolates in the *Trichoderma* species. They found some differences in the genome of two mycoparasitic species such as *T. virens* and *T. atroviride* that contained on a rich of the secondary metabolism-related genes compared with *T. reesei* (Mukherjee et al., 2012). The genomic data was helpful in determining many genes in biocontrol strains of *Trichoderma* such as a comparison between the genome of mycoparasitic strain *T. harzianum* and nonmycoparasitic strain *T. reesei* (Sharma et al., 2017). They detected two acyltransferase encoding genes both of tri3 and tri18 in the gene clusters of the trichothecene biosynthetic (Lindo et al., 2019).

26.9.8 Secretomic

This term is a subset of proteomic including analysis of proteins that secreting. This analysis is useful to identify all the proteins produced by BCA such as *Trichoderma*. Many proteins such as chitinases, glucoamylases, α -1,3-glucanases, α -1,3-glucanases, and proteases were related with mycoparasitism of *T. harzianum* ALL42 (after 24 hours incubated) that induction by adding the cell wall of *Fusarium solani* to culture media (Ramada et al., 2016). They detected many proteins secreted by *T. harzianum* through antagonistic against plant pathogen *Guignardia citricarpa* that were responsible for inducing the plant defense and inhibiting the mycelium growth (de Lima et al., 2017). In addition, they identified a total 626 various proteins group useful for the biomass degradation comprising noncatalytic, accessory, cell-wall-associated proteins, and carbohydrate-active enzymes from the growth of *T. harzianum* in different substrate media (Gómez-Mendoza et al., 2014). The secretome recognized several hydrolytic enzymes comprising β -glucosidases, cellobiohydrolases, α -N-arabinofuranosidases, endoxylanases, endoglucanases, β -xylosidases, xyloglucanases and lignocellulosic that production by *T. reesei* and related with a carbon source such as the sugarcane biomass (Adav et al., 2012; Borin et al., 2015).

26.10 Conclusion

The molecular techniques described in this chapter help to identify different micro-organisms such as: bacteria, fungi, insects, viruses, protozoa, mites and nematodes; The very effective mechanisms against plant pathogens such as parasitism, produce enzymes and secondary metabolites, inducing the defense and resistance of the plant. On the other hand, the omics techniques include proteomics, metabolomics, peptidomics, transcriptomics, phenomics, interactomics, genomics and secretomics, showing a high efficiency in the detection of the metabolome and the expression for several biological control agents.

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Chapter 27

CRISPR/Cas9 mediated genome engineering in microbes and its application in plant beneficial effects

Anshu Alok^{1,2}, Siddharth Tiwari² and Jaspreet Kaur¹

¹University Institute of Engineering & Technology, Panjab University, Chandigarh, India, ²National Agri-Food Biotechnology Institute, Government of India, Mohali, India

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27.1 Introduction

Genetic engineering is extensively used in microbes such as bacteria, yeast and fungi to make them suitable for agriculturally important crops. Various bacteria, are well known to produce various biochemical and byproducts, which helps plants roots to uptake minerals from soil. The biosynthetic pathways of these biochemical or bioproducts are regulated by altering the genetic makeup of microbes. For example, different bacteria (*Pseudomonas*, *Bacillus*, *Azotobacter*, and *Bradyrhizobium*), fungi (*Penicillium* and *Aspergillus*), actinomycetes (*Streptomyces*), and algae are able to solubilize P-metal complex using their siderophore or phosphatase enzymes and ultimately, release bioavailable P (Bargaz et al., 2018). However, few fungal pathogens are well characterized to cause severe diseases in various plants species. To combat or control these nowadays, nonpathogenic microbial antagonists or products derived from their metabolism are used to decrease the specific pathogens (Singh, 2016; Muñoz et al., 2019). *B. subtilis* and other *Bacillus* species is plant beneficial microbe and it profits the plant growth in multiple ways, such as suppressing plants pathogens by releasing antimicrobial compounds, cytokine hormones and volatiles that modify plant hormone homeostasis (Yi et al., 2018). However, the strain improvement by genetic engineering is need for better plant-microbes interaction.

Earlier, homologous recombination, group II intron retrohoming, and multiplex automated genome engineering were used to genome modification in microorganisms (Esvelt and Wang, 2013; Enyeart et al., 2013; Wang et al., 2009; Wang and Church, 2011). However, all this tools were tedious and time consuming. The clustered regularly interspaced short palindromic repeats and its associated proteins (CRISPR/Cas) was explored as genome editing technique in *E. coli* in year 2013 (Jiang et al., 2013). Later on it was efficiently applied into other microbes such as *Saccharomyces cerevisiae* and *Streptomyces* spp. (DiCarlo et al., 2013; Cobb et al., 2015). CRISPR/Cas9 mediated genome modification has also done in *Tatumella citrea*, and another species of the *Enterobacteriaceae* (Jiang et al., 2015). The genome editing

in microbes which are agriculturally important has been also done in rhizobacteria i.e. *B. subtilis* and *B. mycoides*, fungal pathogens i.e. *Neurospora crassa*, *Myceliophthora heterotalica*, *Aspergillus niger*, and *Aspergillus oryzae*, etc (Muñoz et al., 2019).

The CRISPR and its associated proteins are mainly involved into defense mechanism against invader DNA or bacteriophage. There are mainly three types of CRISPR architecture, few of them use single effector proteins such as Cas9 or Cpf1 whereas, others use multiple Cas proteins to target and degrade alien DNA. However, Cas9 endonuclease which belongs to Type II system was the first used in genome engineering due to its simplicity (Jinek et al., 2012; Jinek et al., 2014; Peters et al., 2015). The Cas9 endonuclease was isolated from *Streptococcus pyogenes* which uses two RNA duplex i.e. crRNA and trRNA. However, further this two RNA was simplified and synthesized in vitro, which is well known as single guide RNA (sgRNA). Cas9 has two domains namely HNH- and RuvC-like nuclease domains, which cut sense and antisense strand of dsDNA (Nishimasu et al., 2014). The synthetic gRNA consists 20 nt sequence matching to target DNA or gene, Cas9 and protospacer adjacent motif (PAM) in genome are important key for genome editing. CRISPR/Cas9 has multiple features, among all one of the best important feature is catalytically dead Cas9 (dCas9), which is unable to create break within double stranded DNA (dsDNA) (Qi et al., 2013). The dCas9 has been used for base editing, gene activation/suppression and tagging a specific locus. In this chapter, we will study the journey of CRISPR/Cas9 defense systems towards genome editing, different kind of toolkit for pathways engineering in microbes and its application to make them suitable for better plant microbe's interaction.

27.2 Journey of CRISPR/Cas9 system, immunity to genome editing tool

The CRISPR/Cas9 system is found in few prokaryotes, which enables prokaryotes to respond and to degrade alien DNA. This is one of the mechanism of adaptive immune response of microbes due to insertion of alien nucleic acid by one of following methods i.e. transduction, conjugation, and transformation. The first report of CRISPR architecture was noted in 1985, as a repetitive structure in the 3'-end flanking region of *iap* gene involved in isozyme conversion of alkaline phosphatase in *E. coli*. Remarkably, unlike usual tandem repeats in the bacterial genome, CRISPR repeats were separated by spacers, which is non-repeat DNA sequence (Ishino et al., 1987; Adli, 2018). The term CRISPR (clustered regularly interspaced short palindromic repeat DNA sequences) was named in 2002 by Dr. Schouls group (Jansen et al., 2002). However, at that the functional role of these repeats were unknown. However, in 2005 few groups reported that, these spacers consist homologous sequence of virus and plasmid (Bolotin et al., 2004; Mojica et al., 2000; Pourcel et al., 2005). Soon after this, the role of CRISPR architecture was exposed to offer resistance against invading bacteriophage in *Streptococcus thermophiles* by using Cas genes in 2007 (Barrangou et al., 2007). The Cas genes encode enzymes, which is guided by CRISPR RNAs transcribed by spacer sequences (Brouns et al., 2008). The acquired spacer sequences of CRISPR are extremely similar to each other at PAM (protospacer-adjacent motifs) regions (Deveau et al., 2008) (Fig. 27.1).

All these critical findings laid the platform of discovery of CRISPR/Cas9 genome editing tool. The transformation of CRISPR/Cas system from defense mechanism to genetic editing tool took place, when it was shown that editing could be reprogrammed by changing only 20 nt in the crRNA. Further, the specificity and double-stranded breaks against target were increased by combining crRNA with tracrRNA, which is known as a guide RNA (Jinek et al., 2012). Jinek and coworker also proved that, the Cas9 was RNA guided endonuclease with two nuclease domains responsible for cleavage activity at both strands of DNA. Crystal structure of the Cas9-guideRNA-target DNA complex reveals various facts and strengthened this tools (Nishimasu et al., 2014).

After this, various authors demonstrated the use of CRISPR/Cas9 system based genome editing tool in different microbes. In 2013, Jiang et al., precisely created mutation within the genome of *Streptococcus pneumoniae* and *Escherichia coli* using dual-RNAs. They recovered approximately 100% and 65% *S. pneumoniae* and *E. coli* mutated cells with targeted mutation. Further, the multiplex mutagenesis was also demonstrated using of two crRNAs in *S. pneumoniae* and *E. coli* make this tool important for pathway engineering in bacteria (Jiang et al., 2013). With the time, this tool was also applied into single cells eukaryotes, i.e. *Saccharomyces cerevisiae* for genome engineering. The constitutive expression of Cas9 along with transient gRNA cassette were used to genome editing. However, 100% donor DNA recombination frequency were reported when co-transformation of plasmid carrying gRNA in cells which constitutively express Cas9 (DiCarlo et al., 2013). Shortly thereafter, the functionality of the two-component system was demonstrated in eukaryotes such as human, mouse and zebrafish, indicating the other functions of CRISPR locus gene was supported by eukaryotic endogenous enzymes (Cho et al., 2018; Cong et al., 2013; Hwang et al., 2013; Jinek et al., 2014; Mali et al., 2013).

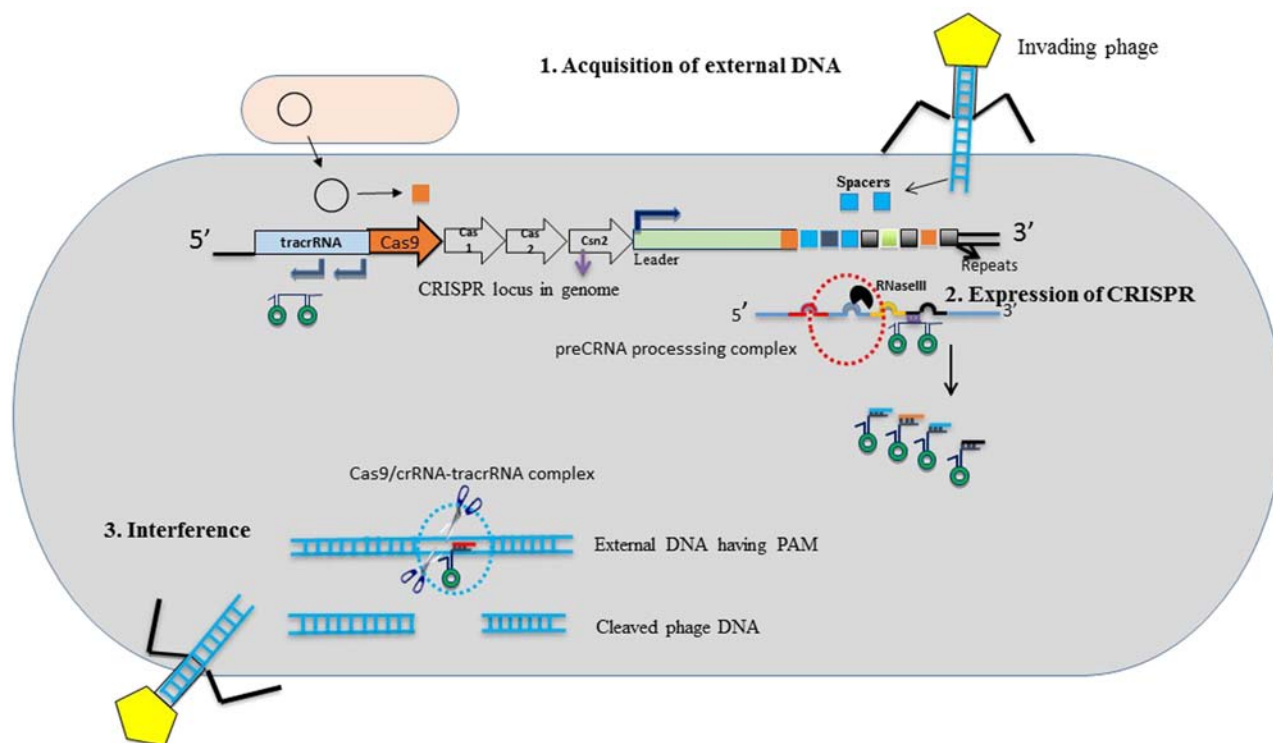


FIGURE 27.1 Natural mechanism of CRISPR immune response. 1. Acquisition of external DNA either by invaders bacteriophage or by conjugation, 2. CRISPR coding and noncoding transcription and 3. interferences with reencounter with same DNA.

27.3 Bioinformatics tools for gRNA selection and off-target effects

The very first step of genome editing is to choose the target region within the DNA of interest. Various online gRNA and off target prediction tools are available for prokaryotic and eukaryotic microbes. Generally, all available tools focused on the following three steps involved.

- Selection of target sites or designing of gRNA,
- Verification of the designed gRNA for possible off-targets, and
- Evaluation of on- and off-target cleavage sites.

For various bacterial species, limited softwares were presently available, i.e. CHOPCHOP (<http://chopchop.cbu.uib.no/>), CRISPR-ERA (<http://crispr-era.stanford.edu/index.jsp>), CRISPy-web (<http://crispy.secondarymetabolites.org/>) and sgRNA scorer2.0 (<https://CRISPR.med.harvard.edu/sgRNAScorerV2/>) etc. The online software CHOPCHOP is also applicable for eukaryotic microbes such as single or multi cell different types of fungi. This tool is available for target and off target prediction of CRISPR/Cas9, CRISPR/Cas9 nickase, CRISPR/Cpf and TALEN. CRISPy-web online tool permits scientists to select a 20 bp region within the DNA or gene of interest. This tool also check potential off-target matches within the genome of selected gRNA and displayed graphically, which can be further transferred to text files (Blin et al., 2016). CRISPR-ERA design gRNA automatically, by genome wide search with different search approach. It allows identification of potential target for Cas9, Cas9 nickase, gene repression and activation of different microbial genomes. Currently, out of different genomes, three microbes, i.e. *E. coli*, *B. subtilis* and *S. cerevisiae* are available in this web tool (Cho et al., 2018).

27.4 Different kinds of toolkit for microbes

Generally, the wild type Cas9 break the dsDNA with the help of gRNA, however few mutants were created for nick only one strand of DNA, which is known as nickaseCas9. Similarly, few mutants of Cas9 protein cannot cleave any strand of DNA, and therefore named as deadCas9. The deadCas9 are fused along with transcriptional activator and suppression which is frequently used in microbes for metabolic engineering.

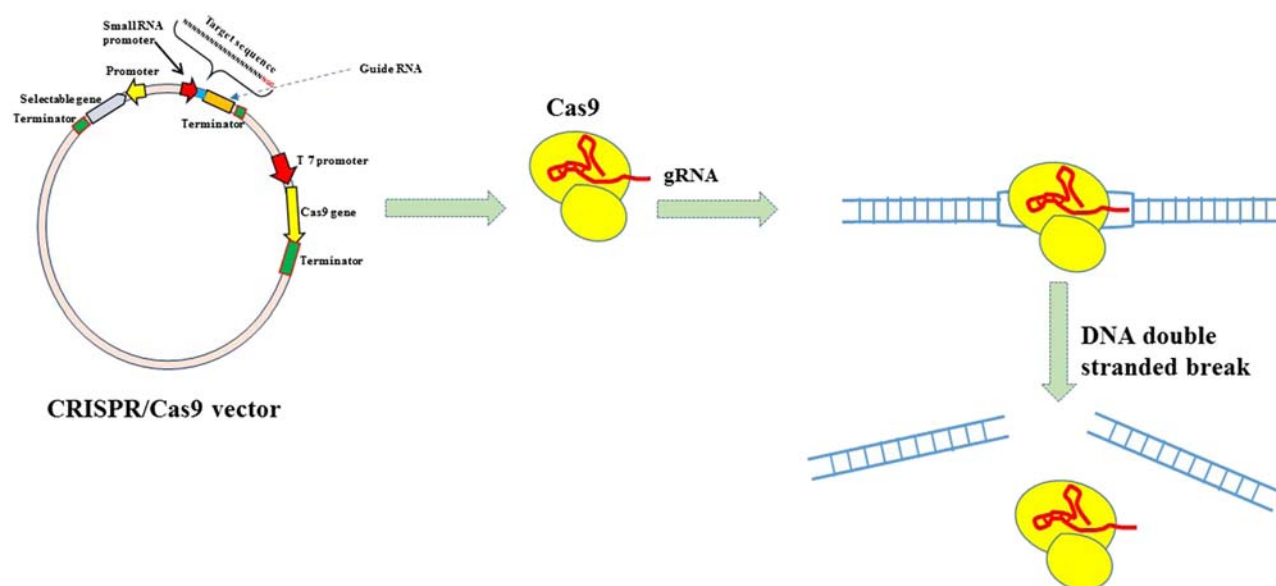


FIGURE 27.2 CRISPR/Cas9 mediated double stranded break within the genome.

27.4.1 CRISPR/Cas9 tool for dsDNA break

The CRISPR/Cas9 tool or vector essentially consist of gRNA and Cas9 gene. The Cas9 and gRNA is a coding and non-coding and therefore these should be regulated by efficient promoters for the function of the CRISPR/Cas9 system. The vector for microbes may be single or two-plasmid system, in which the Cas9 and gRNA gene are situated on different plasmids. Commonly, noncoding RNA promoters are used to regulate gRNA; however, few efficient promoters of coding genes might be used. Various vectors are available for bacteria which use T7 promoter to regulate Cas9. For example, pET-Cas9-NLS-6xHis, pMJ915, pET28a/Cas9-Cys, pMJ806, and pET-NLS-Cas9-6xHis consist T7 promoter (Ramakrishna et al., 2014; Lin et al., 2014; Zuris et al., 2015). The double stranded break using this tool is depicted in Fig. 27.2.

27.4.2 CRISPRi

The CRISPRi tool has been broadly used for metabolic engineering in different microbes such as *E. coli*, *B. subtilis* (Peters et al., 2016), *B. smithii* (Mougiakos et al., 2017), *C. beijerinckii* (Wang et al., 2016) *Mycobacterium* (Rock et al., 2017) and *Streptomyces* (Tong et al., 2015). In this, method the construct carrying dCas9 and gRNA are regulated by their respective promoter in bacterial CRISPR vector. The dCas9-gRNA complex represses the transcription of desired gene by binding to their promoter and hindering RNA polymerase (RNAP) (Qi et al., 2013; Bikard et al., 2013) (Fig. 27.3).

Blocking the elongation of transcript of targeted genes are highly specific and effective, which is further determined by transcriptome analysis. Traditional way of gene regulation with compared to CRISPRi has various drawback and therefore, it is in demand for regulating various genes in a metabolic pathway. The main advantage of the gene suppression via CRISPRi is easy to design and construction of vector. Another advantage of CRISPRi can be used for regulating multiple genes at a time and it can be regulated by inducible promoter which allows conditional repression of targeted gene (Choudhary et al., 2015). The main drawback of CRISPRi is that, it decreases the expression of downstream genes in operons (i.e., polarity) due to the dCas9 block to elongating RNAP (Peters et al., 2015).

27.4.3 CRISPRa

CRISPR mediated transcriptional activation (CRISPRa) using activator domain fused dCas9-sgRNA complex recruit RNA polymerase onto the promoter of targeted gene. In bacterial CRISPRa, especially in *E. coli* dCas9 is attached to ω subunit of RNAP and finally, this complex bind to upstream of the promoter, thus transcription starts. CRISPRa is

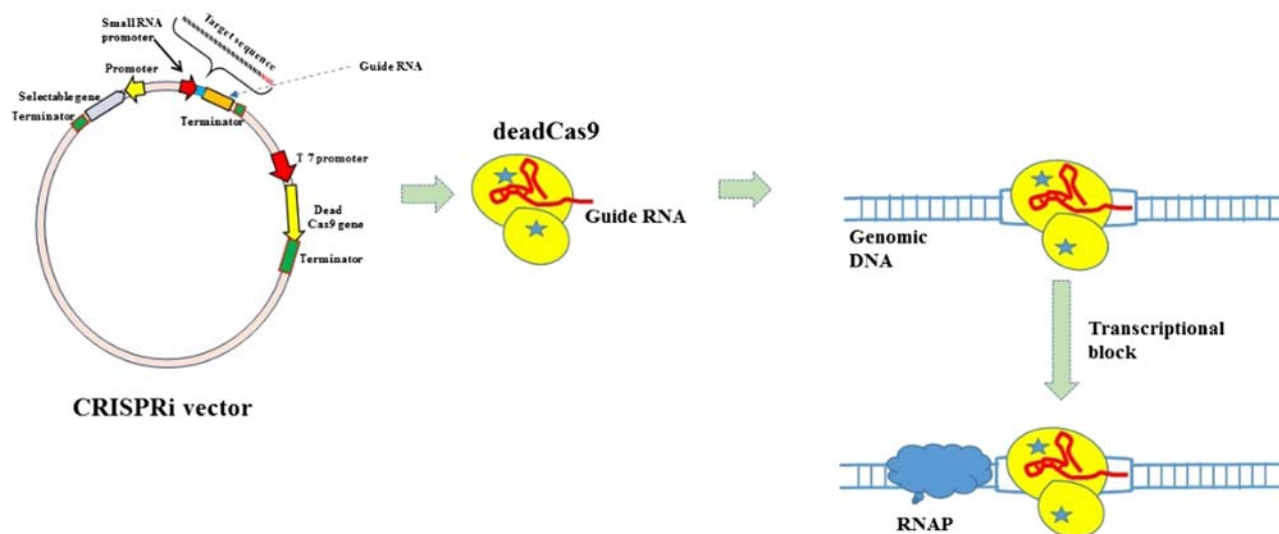


FIGURE 27.3 Mechanism of CRISPRi. The deadCas9 is mutant of wild type Cas9, which is not able to nick or break dsDNA. The gRNA will complementary bind to its target into genome, where deadCas9 will bind and finally block the transcription by inhibiting RNAP complex.

working efficiently for weak promoters. For example, in one of the previous study, showed that 7.2- and 23-fold induction was found when the gRNA was situated 80 and 96 bp upstream of the transcriptional start site (Bikard et al., 2013). This result recommended that a dCas9- ω fusion can activate gene expression when it binds at specific site of promoter (Bikard et al., 2013).

27.5 Genome engineering in agriculturally important bacteria

Few bacterial species are potent for plant growth, which are generally colonized near roots rhizosphere and release siderophores or biochemical byproducts. *B. subtilis* HS3 and *B. mycoides* EC18 are soil borne bacteria present in grass rhizosphere and potato endosphere. These both bacteria showed antifungal, endophytic and plant growth promoting activity, however genetic alteration within genome is difficult by traditional method respectively (Yi et al., 2018). Recently, CRISPR/Cas9 mediated genome editing were performed to create three mutant of *B. subtilis* HS3, while and two mutants of *B. mycoides* EC18 (Yi et al., 2018). 2,3-Butanediol is volatile organic compounds release by *B. subtilis* HS3 well known to stimulate development and growth of grass (Ryu et al., 2003). Acetoin is converted into 2,3-butanediol by enzymes known as acetoin reductase and 2,3-butanediol dehydrogenase (Zhang et al., 2014). The biosynthesis of acetoin from acetolactate was done with help of acetolactate synthase and acetolactate decarboxylase. The 2,3-butanediol dehydrogenase, acetolactate synthase, and acetolactate decarboxylase enzymes are coded by *bdhA*, *alsS* and *alsD* genes respectively (Zhang et al., 2014; Yi et al., 2018). The CRISPR/Cas9 vector namely pJOE8999_galsDHR and pJOE8999_gbdhHR were used to block the either 2,3-butanediol or acetoin or both by targeting *bdhA* and *alsD* genes in this pathways (Yi et al., 2018). By editing two genes in *B. mycoides* EC18, the authors showed that, petrobactin is important and enhance for plant growth via root colonization (Yi et al., 2018).

Metabolic engineering in *E. coli* is broadly achieved by overproduction of important biochemicals such as n-butanol, amino acids, terpenoids, polyketides, and alcohols (Peng et al., 2010; Heo et al., 2017). Now a days various reports has done in which, this fascinating tools were used in *E. coli* to modify genome according to our desired. For example, n-butanol production was enhanced in *E. coli* was done by altering the *citrate synthase* gene in genome using CRISPR/Cas9. The downstream pathways of acetyl-CoA are Krebs cycle and towards acetoacetyl-CoA, a key precursor of butanol biosynthesis. However, the flux of acetyl-CoA of toward Krebs cycle is dominant and hence it should be diverted to acetoacetyl-CoA. Consequently, CRISPR-Cas9 mediated editing of *citrate synthase* gene leads to enhanced n-butanol production (Heo et al., 2017).

β -carotene biosynthetic pathway is well known to be present in few plants species for β -carotene, precursor for vitamin A. Using CRISPR/Cas9 technology, the β -carotene biosynthetic pathway was inserted into genome of

E. coli (Li et al., 2015). They modulated methylerythritol-phosphate and central metabolic pathways to increase β -carotene production. Total 33 genomic alterations were done in *E. coli* and finally 15 mutants were screened with ability to produce 2 g/L β -carotene in fed-batch fermentation (Li et al., 2015). The CRISPR/Cas9 mediated gene editing or insertion can be done without any selection marker, these attribute make this tool unique and important. One more important aspect of this tool is homology based complete pathway insertion in the genome of bacteria. The isobutanol biosynthetic pathway was prepared within 10 kb construct, which was further transferred and integrated into *E. coli* genome via extremely efficient one step markerless CRISPR/Cas9 technique (Heo et al., 2017). Using this tool, isopropanol and natural product pathway was inserted with 5.7 and 14 kb respectively in *E. coli*. They got 100% and 25% integration efficiency for isopropanol and natural product pathway (Bassalo et al., 2016).

27.6 Engineering in fungi and yeast for defense and beneficiary effect on agricultural crops

Various fungal pathogen species such as *Puccinia*, *Fusarium*, and *Blumeria* etc. are devastating for various crops such as wheat, maize, rice and sorghum etc. Various approaches have been used to control the crop loss due to these pathogens for example using nonpathogenic fungus antagonists or fungal products, traditional breeding, and genetic engineering. However, the genetic engineering is the most promising approach for generating fungal tolerant crops. Plant fungal diseases specially can be controlled by inhibition of infection, growth, and reproduction of one fungal species using another (Shishido et al., 2005; Shanahan et al., 1992). The use of CRISPR/Cas9 technology could be used generate mutant nonpathogenic fungal, that can be used as possible competitors against wild type pathogens. Few fungi are act as cell factories and can be used for biosynthesis of agricultural important secondary metabolites (Muñoz et al., 2019). Recently, CRISPR/Cas9 were used to knockout *ura3* gene of *Ganoderma lucidum* 260125 and *Ganoderma lingzhi* (Qin et al., 2017). These fungi produce ganoderic acids which is anti-tumor and anti-metastasis metabolites. The CRISPR/Cas9 mediated editing has done in *Aspergillus* in different study by targeting *polyketide synthase*, *AMP-dependent synthetase*, *Aldo/keto reductase*, *Alcohol dehydrogenase*, *Conidial laccase* and *Orotidine 5-phosphate decarboxylase* (Muñoz et al., 2019).

CRISPR/Cas9 mediated genome editing tool is not only limited to knock out the gene, now a day it is also used promoter engineering. Bacterial transformation via recombination and CRISPR/Cas9 combined together are very useful for multiplex promoter-engineering within yeasts. Till now, various strains of yeast such as *S. cerevisiae*, *Yarrowia lipolytica*, *Pichia pastoris*, *Candida albicans*, *Kluyveromyces lactis*, and *Ustilago maydis* were modified through CRISPR-Cas9 and used as common production strain for valuable products. This method opens a new avenue to explore silent natural products by activating promoter of potential candidate genes. Generally, conventional methods to create mutant yeast strain such as *S. cerevisiae* takes approx. 6 weeks while, using genome editing tool one week is sufficient (Horwitz et al., 2015). Using this tool, we can easily insert DNA having large size, which is useful for quick and stable integration of pathway of desired product or metabolites. For example, 24 kb long construct having six genes were integrated for a commercial product (R, R)-2, 3-butanediol into Ty transposon elements of *S. cerevisiae* (Shi et al., 2016; Stovicek et al., 2017). Out of 6 genes construct 3 from (R, R)-2, 3-butanediol synthesis and 3 genes from xylose consumption pathway. The results state that, nearly 10 copies of 6 genes construct were integrated, which produces 0.31 g/L (R, R)-2, 3-butanediol from 20 g/L xylose (Shi et al., 2016; Stovicek et al., 2017). In another report, CRISPR mediated knockout of multiple genes was used in *S. cerevisiae* in one step (Bao et al., 2014). The Cas9, guide RNA targeting *ATF2*, *GCY1* and *YPR1* genes and numerous HR donors bordered by direct repeats were used for artificial hydrocortisone biosynthetic pathway. The efficiency of homology based integration and gene mutation was up to 100% in yeast (Bao et al., 2014). Baker's yeast, *S. cerevisiae* was used to produce lactic acid using one step CRISPR/Cas9 approach via inserting *Lactate dehydrogenase* gene and knock out 2 endogenous genes i.e. *Pyruvate decarboxylase* genes.

27.7 Conclusion and future perspectives of CRISPR system in agriculture

CRISPR/Cas9 and its variants are frequently used for deletion, insertion, replacement, and regulation of desired gene in microbes. With compare to traditional methods generating bacterial, fungal or yeast mutants are very easy and less time consuming. CRISPR/Cas9 tools use constitutive promoter to regulate Cas9 gene, however in few bacteria the Cas9 protein act as toxic and sensitive due to intracellular release of Cas9. Therefore, the Cas9 gene

TABLE 27.1 CRISPR/Cas9 mediated genome and metabolic engineering in *E. coli*.

CRISPR/Cas9-mediated alteration method	Targeted pathway or gene	Efficiency	References
Programmable DNA looping using Cas9 and deadCas9	Activate endogenous norVW genes	15% looping efficiency, 4.7 kb loop	Hao et al., 2017
CRISPR/Cas9-facilitated multiplex pathway optimization	Xylose metabolic pathway genes	70%	Zhu et al., 2017
CRISPR-Cas12a mediated	Insertion of up to 3 heterologous genes in one round of recombination	20% for 3 gene insertions	Ao et al., 2018
CRISPR-SpCas9	Insertion of up to 3 heterologous genes in one round of recombination	20% for 3 point mutations	Ao et al., 2018
Cas9 mediated editing	Central metabolic pathways and MEP pathway or β -carotene (2.0 g/L)	nearly 100%	Li et al., 2015
Multiple genes repression using CRISPR/deadCas9	(2S)-naringenin, Carbon flux toward malonyl-CoA,	7.4 fold, 421.6 mg/mL (2 S)-naringenin	Wu et al., 2015
CRISPRi	Mevalonate biosynthetic pathway by dnaA, oriC, pyrF, thyA	mevalonate was increased by 41%	Li et al., 2016
CRISPRi	MVA pathway genes, specially acetoacetyl-CoA thiolase gene	Increased production of (-)- α -bisabolol and lycopene	Kim et al., 2016
CRISPRi	Polyhydroxyalkanoate biosynthesis pathway by targeting <i>sadI</i> , <i>sucD</i> , <i>sucC</i> , <i>sdhA</i> , <i>sdhB</i> genes	4HB content in P(3HB-co-4HB) copolymer was increased to 18.4 mol%	Lv et al., 2015
CRISPRi	Pinosylvin, malonyl-CoA pathway Repression of <i>fabD</i> gene	47.49 mg/L pinosylvin was produced from glycerol	Liang et al., 2016

should be regulated by inducible promoters for metabolic engineering in microbes. Multiplexing is now become very important for multiple or complex pathways for a desired product for large scale industrial applications (Table 27.1).

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Chapter 28

Understanding the molecular basis of the tripartite interaction between host-pathogen-bioagent through proteomic approach

S.R. Prabhukarthikeyan, U. Keerthana, Manoj K. Yadav, S. Raghu, S. Aravindan, Mathew S. Baite and P.C. Rath
Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, India

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28.1 Introduction

Proteomics is an ideal tool for illumination of plant microbe interactions. First, it provides a broad summary of the proteins produced by both partners during their constant signal exchange and, in particular, enables the effect on gene product networks of gene knockouts, additions and specific growth states to be determined. Second, it allows the revealing of signal transduction pathways by following phosphorylation changes of proteins that are important for protein function (Peck et al., 2001). Understanding the plant responses to biotic stresses at the genomic level provides an essential foundation for studying disease resistance in host plants. Genome wise identification of genes regulated by pathogen and biocontrol agents has manifold significance. First, it provides a more comprehensive understanding of the transcriptional responses. Second, it provides a starting point for elucidating the role of individual genes in response to multiple interactions, which will be of great value in crop plant engineering. Third, it aids in the identification of stress responsive promoters and responsible cis elements in both basic as well as applied aspects. DNA microarray provides a high throughput means of analyzing gene expression, which has been in use to study patterns of gene expression in response to stress (Seki et al., 2003).

The three-way interactions involving biocontrol, plant and fungal pathogen have received less attention in comparison to the “simple” two-partner systems (i.e. plant–pathogen, plant–antagonist or pathogen–antagonist) (Harman et al., 2004; Suzuki et al., 2004). However, the three-way interaction between host-pathogen-PGPR provides a complete view of defense mechanisms mediated by biocontrol agents. The proteome analysis with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used to study the changes in the protein expression of tissues under different stresses (Komatsu et al., 2004). The interactions between plant and PGPR strain KH-1 induced differential proteins which played the major role in the growth promotion of rice plants (Saveetha, 2009). Marra et al. (2006) investigated the complex interactions between bean plants, fungal pathogens (*Botrytis cinerea*, *Rhizoctonia solani*) and *Trichoderma* through proteomic approach, to analyze the differentially expressed proteins. Recently, Prabhukarthikeyan et al. (2017) identified 12 differentially expressed proteins during the interactions between *Curcuma longa* - *Pythium aphanidermatum* – *P. fluorescens* through proteomic analysis.

28.2 Genesis of proteomic technology in crop improvement

Recently, DNA microarray and proteomics techniques have been used to analyze expression patterns of highly complex mixtures of mRNA or proteins and to explore gene functions at the genomic level in different tissues or stress conditions. These approaches examine whole gene expression patterns in certain stressed environments that provide a “global” snapshot of genetic factors involved or associated with plant defense mechanisms. The DNA genomic sequence has been considered by some as a basic framework for further investigations into mechanisms of gene action (Whitelegge, 2002). Because the amount of protein is often not predictable from the amount of mRNA produced in the cell and post translational modifications such as phosphorylation and glycosylation can be vital for cellular functions, the study of proteins is crucial to judiciously examine their levels and activities at a given time in a tissue or in response to a certain environment or condition. The induction of PR proteins has been detected in various plant species and PR-proteins have been classified into 17 families based on sequence similarity and enzymatic properties (Van Loon et al., 1998). Various genes that encode defense-related proteins have been inserted into plants and crops to enhance resistance to pathogens (Grover and Grwthama, 2003).

28.3 Protein profiling

Although the concept of the “proteome” is currently receiving considerable attention, identification of specific proteins is an established technique whereby isolated proteins are first separated by their isoelectric point (pI) in the first dimension of electrophoresis and then separated by their molecular weight in the second dimension using SDS-PAGE. With two dimensional electrophoresis (2-DE), ~2000 proteins can theoretically be separated and displayed in a single gel (O’Farrell, 1975).

28.4 Protein identification

In the 1990 s, new protein identification methods based on mass spectrometry dramatically improved the efficiency (Yates, 1998). For this method, the protein of interest, displayed in the 2-DE gel, is either enzymatically or chemically cleaved into several peptides and the mixture is analyzed by mass spectrometric techniques. This group of peptides is referred to as a peptide mass fingerprint (PMF). Results obtained from mass spectrometric analysis is compared to virtual fingerprints obtained from theoretical cleavage of protein sequences stored in data bases for candidate protein identification. Using computer technology, proteins can be identified with peptide mass fingerprinting with the sequenced protein compared with known proteins in appropriate databases (Gevaert and Vanderkerckhove, 2000). Recently, the DNA sequence from more than 30 organisms including *Escherichia coli*, *Arabidopsis thaliana* and *Oryza sativa* has been determined. Tandem mass spectrometry (MS/MS) is the preferred instrument for high-throughput protein identification (Havilo et al., 2003). An Electro Spray Quadrupole Time of Flight (ESI-Q-TOF) instrument determines sequence information of the peptide from trypsin digestion by providing MS/MS of peptide mass and fragmented ion mass of individual peptides. Two methods are used in analysis of protein identification with the MS/MS spectrum. The first method converts the MS/MS spectrum into a probable amino acid sequences (*de novo* sequencing) while the second method involves searching databases containing theoretical MS/MS spectra. The MS/MS analysis method can be used in various organisms whose DNA sequence data base are not well developed because this analysis method relies on amino acid sequence of peptides. Moreover, the quality of protein identification of MS/MS analysis is generally regarded to be more acceptable than that of PMF whose method relies only on mass similarity of peptide fragments of a protein (Heazlewood et al., 2003).

28.5 Records of interactions studies

Studies published so far on plant-pathogen interactions have been mainly focused on the molecular changes related to pathogen attack and/or plant response (Suzuki et al., 2004). Several signal molecules and defence factors have been identified in plant as well as virulence and avirulence factors in microbes. Nevertheless, the molecular bases of multiple-player systems that may produce beneficial effects on plant health are largely unknown. Moreover, the influence that a biocontrol agent may have on the interactions between a plant and a pathogen has not yet been investigated by using proteomics, while this technique clearly represents an effective tool to analyze such biological processes (Woo et al., 2006). Functional genomics combined with bioinformatics provides an overall picture of the metabolic status of an organism in a given moment or condition (Zhu et al., 2003). In particular, proteomics permits a large-scale analysis

of protein production and in recent years has been widely used to investigate protein profiles produced from diverse interaction conditions and physiological states of cells and tissues (Zhu et al., 2003), especially in pathology-related research (Lim and Elenitoba-Johnson, 2004).

ABC-type transporters, membrane and regulatory proteins, nodule-specific proteins and nutrient stress-specific proteins were identified in legume-rhizobium interactions (Rolfe et al., 2003). Grinyer et al. (2005) pictured the effect of cellular proteins of *T. atroviride* mycelia on *R. solani* cell walls through their differential protein profiling approach. Zhou et al. (2006) reported the expression of transcript levels of many pathogenesis-related (PR) genes following *F. graminearum* infection in wheat. Different classes of PR proteins including PR-1, PR-2 (β -1, 3 glucanases), PR-3 (chitinases), PR-5 (thaumatin-like protein) and PR-9 (peroxidases) were found to be induced within 6–12 h of inoculation. The antioxidant functional proteins (ascorbate peroxidase and glutathione transferase) also found to be upregulated in the above said interactions.

Zinc finger protein, pathogenesis-related/seed storage/lipid transfer protein (LTP) family protein, leucine-rich repeat family protein/protein kinase family protein, protease inhibitors/seed storage/lipid transfer protein (LTP) family protein and auxin response protein were upregulated and F-box family protein, RNA-binding protein, putative cytochrome P450, protein kinase family protein, leucine-rich repeat family protein/protein kinase family protein, CBL-interacting protein-kinase 9 and disease resistance protein (TIR-NBS class) are found to down regulated in plant-parasitic nematodes interactions (Fuller et al., 2007). Studies on Cocoa-witches broom interaction revealed the expression proteins like leucine-rich protein, β -1, 3 glucanase, cysteine proteinase, cytochrome P450 and endo-1,4-beta-glucanase (Leal et al., 2008). Karthiba (2012) studied the three-way interaction of rice treated with biocontrol agents challenged with sheath blight pathogen and identified 30 differential expressed proteins. Out of 30 differentially expressed proteins, 18 proteins were up regulated in pathogen but down regulated in PGPR - pathogen interaction, 7 proteins were found to be up regulated in both pathogen and PGPR - pathogen interaction, 3 protein spots were down regulated in pathogen and 2 protein spots having changes in position comparing control, pathogen alone & PGPR alone. Similarly, Senthil (2013) identified the *Chaetomium globosum* mediated protein and their mechanism against chilli damping of disease through proteomic approach. The proteins expressed in the mango fruit tissues collected under three conditions viz., healthy control, pathogen inoculated and interaction of pathogen - PGPE interaction were analyzed by 2D-PAGE and MALDI-TOF. Overall, the twenty two differentially expressed proteins were categorized into 7 different classes based on their abundance ratio (Archana, 2014).

28.6 Applications of proteomic technology

The 2-DE has been used to study genetic differentiation of 11 wheat populations originating from a single population that evolved independently during eight years of cultivation in different locations (David et al., 1997). Of the 48 proteins used to investigate population differentiation, 15 showed significant differences at $P < 0.05$ level. Populations that evolved independently at the same location showed similar differentiation, even though their cultivation methods were different. They concluded that natural selection acted strongly on the genetic composition of the populations. Variations in gene expression have been studied during development and in response to various treatments, and this has led to identification of proteins whose expression is regulated under the conditions described above. Imin et al. (2001) displayed over 4000 proteins of rice anthers at the young microspore stage, representing 10% of the estimated total genomic output. They constructed rice proteome reference maps that could be used as a model of anther proteomes for related species.

Konishi et al. (2001) compared protein expression patterns in rice after inoculation with the blast fungus, *Magnaporthe oryzae*, under different levels of nitrogen fertilization. They found that RuBisCO (rubulose -1, 5-bisphosphate carboxylase/oxygenase) increased proportional to the amount of nitrogen applied and the rice thaumatin-like protein (PR-5) was induced 120 h after blast fungus inoculation. The advantage of displaying several hundred or more gene products on a single gel can be used to examine differences in protein expression patterns between mutants and their corresponding wild types. By comparing 2-DE patterns of the wild type and the mutant, the effect of the mutation can be evaluated and characterized at the protein level. Damerval and Le Guilloux (1998) examined the effect of the O2 mutation on maize protein expression during grain development by 2-DE in seven different pairs of near-isogenic lines. The abundance of 36 polypeptides was found to be modified in the seven backgrounds. Protein products of the known target genes, such as zeins, B-32 protein and pyruvate orthophosphate dikinase were present in greater amounts in normal lines than in mutants. Moreover, enzymes belonging to various metabolic pathways were tentatively identified, most of which were not previously known to be affected by the O2 mutation. They concluded that the O2 gene could act as a connecting regulatory gene for different pathways of grain metabolism. With developed

technologies in proteomics, rice researchers have detected and identified specific proteins that are expressed in certain conditions such as biotic and abiotic stress, different cells, tissues and developmental stages (Koller et al., 2002). Salekdeh et al. (2002) analyzed changes in the rice leaf proteome during drought stress. More than 2000 proteins were separated reproducibly and 50% of the total was quantified by gel image software. Forty-two proteins showed significant changes in their abundance in response to drought stress. Previously known functions of these proteins were involved in photosynthesis, cell elongation, anti-oxidant metabolism or lignification.

28.7 Conclusion

Understanding of the molecular basis of the tripartite interaction will help us to develop disease management strategies through new biotechnological methods and enhances the crop productivity in a sustainable manner.

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Chapter 29

Frankia and the actinorhizal symbiosis

Didier Bogusz and Claudine Franche

UMR DIADE, Rhizogenèse, Institut de Recherche pour le Développement (IRD), Montpellier, France

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29.1 Introduction

The genus *Frankia* comprises gram-positive actinobacteria belonging to the clade Frankiaceae in the order Actinomycetales (Normand et al., 2014). These filamentous microorganisms are characterized by slow growth and high genomic GC content. They are differentiated into three cell types, vegetative hyphae, vesicles and sporangia containing spores. Among actinomycetes, the closest relative to *Frankia* is the non-sporulating, rod-shaped, cellulolytic thermophile *Acidothermus cellulolyticus*. *Frankia* can occupy two distinct ecological niches, the soil and root nodules formed on non-leguminous plants named actinorhizal plants (Sellstedt and Richau, 2013). Actinorhizal plants belong to 25 plant genera, mainly trees and shrubs (with the exception of the perennial herb *Datisca*), representing eight plant families in the orders Fagales (*Betulaceae*, *Casuarinaceae*, and *Myricaceae*), Rosales (*Rosaceae*, *Eleagnaceae*, and *Rhamnaceae*) and Cucurbitales (*Datisceae* and *Coriariaceae*) (Dawson, 2007). *Frankia* can fix atmospheric nitrogen in aerobic free-living conditions as well as in the cortical cells in actinorhizal root nodules that protect the nitrogenase complex from oxygen inactivation. Together with legumes (Fabales), actinorhizal plants form a single nitrogen-fixing clade (Doyle, 2011).

Unlike rhizobia, which were obtained in pure culture toward the end of the 19th century, the first successful isolation of an effective *Frankia* strain from nodules of *Comptonia peregrina* was only achieved in 1978 (Callaham et al.) and numerous strains remain uncultured to this day. Molecular studies of actinobacteria have faced different problems. Many *Frankia* strains are characterized by a slow growth rate with low biomass yield. The lack of a genetic system for *Frankia* together with the difficulty in obtaining targeted mutants have been two further major obstacles to the functional analysis of candidate genes involved in specific functions such as regulation of nitrogen fixation or symbiotic interaction with their host plants. However, since 2007, the elucidation of numerous *Frankia* genomes in parallel with the development of “omics” including transcriptomic, proteomic and metabolomic approaches, have enabled significant advances in our knowledge of these actinobacteria (Normand et al., 2007a; Tisa et al., 2013).

Genome comparative analyzes revealed the potential of *Frankia* with respect to metabolic diversity, natural product biosynthesis and stress tolerance (Normand et al., 2007b; Udworthy et al., 2011; Rehan et al., 2016). Some *Frankia* strains were shown to have a strong potential for bioremediation and, when they are in a symbiotic form, increase the

fitness of actinorhizal plants growing in environmentally harsh environments including under high salinity, heavy metal and extreme pH conditions (Dawson, 2007; El Dein Abdel-Lateif et al., 2018). Actinorhizal plants are economically and ecologically important for desert reclamation, regeneration of poor and degraded soils, agroforestry, sand dune stabilization and protection of coastal areas (Zhong et al., 2010; Diagne et al., 2013; Kucho et al., 2018). Among the best known actinorhizal plants are the temperate tree species *Alnus glutinosa* and, in tropical arid and semi-arid areas, *Casuarina equisetifolia*.

Some original features of the symbiotic life of *Frankia* attracted the interest of the research community studying symbiotic nitrogen fixation (Bogusz and Franche, 2012). Compared to the gram-negative unicellular Rhizobia, *Frankia* is a filamentous gram-positive actinobacteria. Concerning the nodules, the main difference in the anatomy of an actinorhizal nodule and a legume nodule is the location of the vascular bundles, which are central in nodules resulting from *Frankia* infection, and lateral in rhizobium-infected nodules. Due to this organization, actinorhizal nodules have been compared to symbiotic lateral roots. Comparing the diverse range of signals and molecular mechanisms deployed by a nitrogen-fixing microsymbiont at the early stages of the interaction with the host plants is expected to help achieve the ultimate goal, i.e. the transfer of symbiotic nitrogen fixation to cereals.

The aim of this chapter is to highlight the most recent findings concerning *Frankia* growing in free-living conditions or as a symbiotic partner of its actinorhizal host.

29.2 The microorganism *Frankia* in a free-living stage

29.2.1 General features

Frankia is a pleiomorphic actinobacterium that develops two specific structures, vesicles and spores, that are critical for its survival in the rhizosphere (Sellstedt and Richau, 2013). Vesicles are specialized nitrogen-fixing cells that differentiate at the tips of growing vegetative hyphae when exposed to nitrogen-limited conditions and oxygen. They are surrounded by a laminated lipid envelope containing a mixture of hopanoid lipids which are pentacyclic bacteriohoppanes. The thickness of the vesicle envelope increases with an increase in O₂ and helps to maintain partial oxygen pressure at levels that are not labile for the dinitrogenase complex (Berry et al., 1993; Dobritsa et al., 2001). Most strains develop spores in environmental stress conditions. The spores are formed in sporangia at the mycelial tips and are thought to contribute to the dissemination and survival of the microorganism. Whereas some *Frankia* strains, referred to as «Sp-», differentiate spores only in pure culture, others, called «Sp+» strains, can also sporulate in actinorhizal nodules (Cotin-Galvan et al., 2016).

29.2.2 Taxonomy

Since the first report of the infective *Frankia* strain isolated from nodules of *C. peregrina* (Callaham et al., 1978), hundreds of *Frankia* isolates have been described. Individual strains can nodulate actinorhizal plants from different orders, thus implying that the host origin is not always a determining characteristic for strain classification. On the basis of cross-infectivity studies and genotypic and phylogenetic approaches, three cohesive *Frankia* clusters with distinct host ranges have been defined, plus a large fourth group of «atypical» strains (Clawson et al., 2004; Normand and Fernandez, 2009; Pozzi et al., 2018). Cluster I *Frankia* include strains that nodulate members of the hamamelid families *Betulaceae*, *Casuarinaceae* (not *Gymnostoma*) and *Myricaceae*. Cluster II *Frankia* mostly include non-isolated endophytes that are typically associated with members of the *Coriariaceae*, *Datiscaceae*, *Rosaceae* and *Ceanothus* of the *Rhamnaceae*. Only one *Frankia* cluster II strain isolated from the nodules of *Coriaria myrtifolia* has been cultured so far (Gtari et al., 2015). This cluster II strain has a basal position in the phylogeny of *Frankia* strains and appears to be the most ancient group. Cluster III is still poorly known; strains appear to nodulate *Eleagnaceae*, *Rhamnaceae* (not *Ceanothus*) and *Gymnostoma* belonging to the family *Casuarinaceae*. Despite being isolated from actinorhizal nodules, the atypical *Frankia* strains in cluster IV are no longer able to fix nitrogen and/or re-induce nodules on the root system of their initial host plant. After the recent analysis of 75 *Frankia* strains representing the different clusters, 28 groups of closely related strains called operational taxonomic units (OUT) were recently recognized: 12 OTU belonged to cluster I, two to cluster II, eight to cluster III and six to cluster IV (Pozzi et al., 2018).

Based on their different requirements with respect to *Frankia* strains, actinorhizal plants have been divided into promiscuous and non-promiscuous species (Valdes, 2008). Non-promiscuous plant genera include *Allocasuarina*, *Casuarina*, *Eleagnus* and *Hippophae*, whereas in greenhouse conditions, *Myrica* can be nodulated by most *Frankia* strains. Although specific, the genera *Alnus* and *Gymnostoma* can sometimes be nodulated by *Frankia* strains from the

Eleagnaceae infective group. These data suggest that the plant-actinobacterium interaction may be less specific than the legume-*Rhizobium* symbiosis.

29.2.3 Frankia genomes

The genomes of *Frankia* strains belonging to the four different clusters have been sequenced (Table 29.1). One surprising result was the wide variation in genome sizes that ranged from 5 to 10.5 Mbp. Marked differences were observed in cluster I, with genome sizes ranging from 5.0 to 5.6 Mbp for *Casuarina*-infective strains and from 7.5 to 7.7 Mb for the other strains. So far, cluster II genomes are consistently in the lower size range, ranging from 5.3 to 5.6 Mb. Cluster III genomes range from 7.6 to 10.5 Mb similar to cluster IV genomes that range from 6.8 to 10 Mbp (Table 29.1).

These variations in genome size have been suggested to reflect differences in saprotrophic potential and to influence the host range. In cluster I, strains CcI3 and ACN14a were sequenced in 2007 (Normand et al., 2007a,b). The genome of the narrow host range strain CcI3 isolated in Australia from *Casuarina* sp. is 5.4 Mbp in size and that of the medium host-range *Frankia* ACN14a infective of *Alnus* sp. is 7.5 Mbp. CcI3 is not detected in soils in the absence of a suitable host plant, indicating that this strain depends on the host tree for its propagation in soils. In contrast, ACN14a is widely present in soils even when there is no host plant, probably as the result of its ability to adapt to new environments (Normand et al., 2007a).

Frankia Dg1 in cluster II is the obligate symbiont of *D. glomerata*. So far, all attempts to grow this strain in pure culture have failed and the inoculation of the host plants must be performed with crushed nodules containing Dg1 in infected cells. Its small genome consists in one circular chromosome 5.3 Mbp in size, with 4202 sequences encoding proteins and 325 pseudogenes (Persson et al., 2011). It has been suggested that the genome of Dg1 might have been reduced or degraded during evolution, thus accounting for the difficulty encountered in isolating and growing the

TABLE 29.1 *Frankia* strains with available genome sequences.

Taxonomy	Strain	Initial plant host	Geographical origin	Genome size (Mbp)	Reference
Cluster 1	ACN1ag	<i>A. glutinosa</i>	Canada	7.5	Swanson et al., 2015a
	ACN14a	<i>Alnus viridis</i> subsp. <i>crispa</i>	Canada	7.5	Normand et al., 2007b
	Allo2	<i>Allocasuarina verticillata</i>	Egypt	5.3	Oshone et al., 2016
	Avcl1	<i>A. viridis</i> subsp. <i>crispa</i>	Canada	7.7	Swanson et al., 2015b
	BMG5.23	<i>Casuarina glauca</i>	Tunisia	5.3	Ghodhbane-Gtari et al., 2014
	BR	<i>C. equisetifolia</i>	Brazil	5.2	D'Angelo et al., 2016b
	CcI3	<i>Casuarina</i> sp.	Australia	5.4	Normand et al., 2007b
	CcI6	<i>Casuarina cunninghamiana</i>	Egypt	5.6	Mansour et al., 2014
	CeD	<i>C. equisetifolia</i>	Senegal	5.0	Ngom et al., 2016
	KB5	<i>C. equisetifolia</i>	Australia	5.5	Pesce et al., 2017
	ThR	<i>C. cunninghamiana</i>	Egypt	5.3	Hurst et al., 2014
	QA3	<i>Alnus nitida</i>	Pakistan	7.6	Sen et al., 2013
	BMG5.30	<i>Coriaria myrtifolia</i>	Tunisia	5.8	Gueddou et al., 2018
	Cp1IT	<i>C. peregrina</i>		7.6	Callaham et al., 1978
Cluster 2	Dg1	<i>Coriaria nepalensis</i>	Pakistan	5.3	Persson et al., 2011
	Dg2	<i>Datisca glomerata</i>	United States	5.92	Van Nguyen et al., 2016
	BCU110501	<i>Discaria trinervis</i>	Argentina	7.9	Wall et al., 2013
Cluster 3	BMG5.12	Soil	Tunisia	7.6	Nouioui et al., 2013
	Cc1.17	<i>Colletia cruciata</i>		8.4	Swanson et al., 2017
	CcI49	<i>C. cunninghamiana</i>	Egypt	9.76	Mansour et al., 2017
	EAN1pec	<i>Eleagnus angustifolia</i>	U.S.A.	9.0	Normand et al., 2007b
	EI5c	<i>Eleagnus angustifolia</i>		6.6	D'Angelo et al., 2016a
	G2	<i>C. equisetifolia</i>	Guadeloupe	9.5	Nouioui et al., 2016
	R43	<i>C. cunninghamiana</i>	Australia	10.45	Pujic et al., 2015
	BMG5.36	<i>Coriaria myrifolia</i>	Algeria	11.2	Gueddou et al., 2017
Cluster 4	CN3	<i>Coriaria nepalensis</i>	Pakistan	10.0	Ghodhbane-Gtari et al., 2013
	DC12	<i>Datisca cannabina</i>	Pakistan	6.8	Tisa et al., 2015
	EUN1h	<i>Eleagnus umbellata</i>	Tunisia	9.9	Gueddou et al., 2017
	NRRL	<i>Morella californai</i>	United States	9.4	Gueddou et al., 2017

symbiont of *D. glomerata*. The genome of a second strain isolated from California is now available for this cluster (Van Nguyen et al., 2016).

In contrast, cluster III strains can be isolated, grown in free-living conditions and are globally distributed in soils with or without their actinorhizal host. These actinobacteria have a broad range of hosts and can associate with five actinorhizal plant families belonging to the orders Fagales and Rosales. They have the greatest metabolic versatility and many strains belonging to this cluster have adapted to a wide range of soil types and climates. *Frankia* EAN1pec, a strain isolated from *Eleagnus angustifolia*, was the first representative of this cluster to be sequenced. The genome is 9 Mbp in size, with 7976 putative coding sequences (Normand et al., 2007a). Comparative analysis of four *Frankia* strains isolated from *Eleagnus* sp. suggests that the genomes have deleted and acquired a large number of genes in order to adapt to a wide range of hosts and environments (Kučho et al., 2014). Since the host range of strains isolated from *Alnus* is not as broad, a similar but less pronounced phenomenon is expected.

29.2.4 *Frankia* mutants and genetic transformation

The development of genetic tools for *Frankia* has progressed slowly and remains a major obstacle to functional analyzes of actinobacterial genes and to analysis of the molecular dialog between the symbiont and the host plant (Santi et al., 2013; Froussart et al., 2016a). Although several attempts have been made to develop shuttle vectors from native *Frankia* plasmids (John et al., 2001; Lavire et al., 2001; Xu et al., 2002), some difficulties remain to be overcome before genes can be transferred and targeted mutations in the actinomycete achieved.

To produce *Frankia* strains amenable to genetic transformation, mutagenetic treatments with ethyl methanesulfonate (EMS) were first optimized on the strains Cc1.17 and Eullc (Myers and Tisa, 2004). Antibiotic-resistant (lincomycin, tetracyclin and ampicillin) and antimetabolite (5-fluorouracil) resistant mutants were obtained at a frequency of 3×10^{-3} to 4×10^{-5} . Since very few antibiotic resistant mutants are available in *Frankia* strains, these mutants are valuable potential recipient strains for future conjugation experiments. A chemical mutagenesis of CcI3 hyphae fragments was developed next (Kakoi et al., 2014). This procedure enabled the isolation of uracil auxotroph actinorhizal colonies. Some mutants with altered pigmentation were also obtained and one colony had reduced nitrogen-fixation ability. This procedure represents the first step towards isolation of loss-of-function mutants in CcI3. In 2017, Kucho et al. (2017) reported isolation of candidate nitrogen-fixing mutants of *Frankia* CcI3 after mutagenesis experiments with 1-methyl-3-nitro-1-nitrosoguanidine or gamma X rays. Eleven mutants had lost their ability to fix nitrogen. Some of them had fewer vesicles and their hyphal morphology was altered. Gene transcription analyzes also revealed that the expression of sequences necessary for nitrogen fixation activity was reduced. Genome sequencing of eight mutant strains revealed the presence of 20–400 mutations. Some mutations were localized in known genes related to nitrogen fixation, such as the nitrogenase structural genes *nifD* and *nifH*, thus confirming the link between the altered phenotype and the mutagenic treatment.

Potential transformants of *Frankia* CcI3 were reported in 2009 following electroporation experiments with a vector carrying a tetracyclin resistance gene with codon usage similar to that of *Frankia*, and driven by a native promoter (Kučho et al., 2009). Antibiotic selection in liquid culture enabled the growth of tetracyclin resistant hyphae. However, analysis of the genomic DNA from the putative transformants revealed that the transformation was unstable and that most of the gene constructs were not integrated into the host.

The most recent paper concerning the genetic transformation of *Frankia* was published in 2019 (Pesce et al., 2019). Following the conjugation of *Frankia* CcI3 with the *Escherichia coli* strain BW2927 containing the plasmid pHKT1 expressing the green fluorescent protein (GFP) reporter gene, some transformants exhibiting GFP fluorescence were obtained at a frequency of 4×10^{-3} . As determined following PCR experiments, the plasmid was maintained in the hyphae for over three years and was successfully introduced in three other *Frankia* strains belonging to clusters I, III and IV. The efficiency of plasmid transformation ranged from 1.7×10^{-2} to 3×10^{-4} . It remains to be established if the procedure can be easily repeated in other laboratories.

29.2.5 “Omics” for a better knowledge of *Frankia*

Whereas many *Frankia* strains have been isolated, transcriptomic and proteomic studies have focussed on limited number of strains, the most widely studied actinobacterial strains being ACN14a and CcI3, the respective microsymbionts of *A. glutinosa* and *C. glauca* (Table 29.2) (Benson et al., 2011).

In 2011, Bickhart and Benson studied the transcriptomes of *Frankia* CcI3 as a function of the source of nitrogen (ammonium-supplemented growth conditions versus nitrogen fixing conditions) and culture age (three days versus

TABLE 29.2 Main “Omics” approaches developed in *Frankia*.

“Omics” approach	<i>Frankia</i> strain	Objectives of the studies	References
Transcriptomic	ACN14a	Study of <i>Frankia alni</i> symbiotic transcriptome Response of <i>Frankia</i> to plant phenolic compounds Response to nitrogen starvation Genes differentially expressed during the early stages of the symbiotic process with <i>A. glutinosa</i>	Alloisio et al., 2010 Popovici et al., 2010 Lurthy et al., 2018 Pujic et al., 2019
	Cc13	<i>Frankia</i> gene expression in different physiological conditions <i>Frankia</i> transcriptome under nitrogen fixing conditions	Bickhart and Benson, 2011 Lee et al., 2013
Proteomic	Cc16	Mechanism of salt tolerance in <i>Frankia</i>	Oshone et al., 2017
	Dg1	Genes expressed in symbiosis	Persson et al., 2015
	ACN14a	Biochemical events linked with nitrogen fixation Analysis of the PEG-responding desiccome Proteins displaying differential accumulation during the symbiotic dialogue with <i>A. glutinosa</i>	Alloisio et al., 2007 Ghedira et al., 2017 Pujic et al., 2019
	ACN14a & EaN1pec	Proteins differentially expressed by phenolic compounds Proteome of field-collected <i>Frankia</i> strains	Bagnarol et al., 2007 Mastrorunzio and Benson, 2010
	BMG5.1	Exoproteome analysis in response to root exudates <i>Frankia</i> proteome linked to host recognition	Miotello et al., 2017 Ktari et al., 2017a,b
	Cc13	Exoproteome of <i>Frankia</i> in culture and symbiosis	Mastrorunzio et al., 2009
	Cc13 & EAN1	Identification of secondary metabolites	Udwary et al., 2011
	Cc16	Mechanism of salt tolerance in <i>Frankia</i>	Oshone et al., 2017
Metabolomic	EaN1pec	Mechanism of lead resistance in <i>Frankia</i>	Furnholm et al., 2017
	Hsl18 & Hsl110	Identification of salt stress-induced proteins	Srivastava et al., 2017
	ACN14a	Organic acid metabolism	Carro et al., 2016

five days). Comparative analyzes of the transcriptomes revealed more heterogeneity in gene expression between the three-day-old and the five-day-old cultures than between three-day-old cultures growing with or without ammonium. A high level of expression of a gene encoding a transposase ORF was found in the five-day-old and nitrogen-fixing cultures, suggesting that the stresses resulting from N-starvation and aging induce genome modifications such as gene duplication or deletion, thus contributed to the evolution of *Frankia*. Additional data came from a study performed in 2013 by Lee et al. Whole gene expression profiling revealed a two-fold increase in the expression of the nitrogen fixation-related genes when Cc13 was grown in the absence of ammonium. Although biochemical analyzes demonstrated that nitrogen-starved *Frankia* cells produced 1.7-fold more exopolysaccharides (EPS) than Cc13 hyphae growing in medium supplemented with ammonium, microarray data did not provide evidence for modification of gene expression that could account for the increase in EPS.

A proteome approach was used on *F. alni* ACN14a to study the physiology of *Frankia* and to analyze the biochemical events associated with the switch from N₂-replete to N₂-fixing conditions (Alloisio et al., 2007). A total of 126 highly abundant proteins were studied in a pure culture of ACN14a and found in three different compartments, cytosolic, membrane associated and excreted in the culture medium. Upregulation of stress proteins and proteins involved in N assimilation was observed under nitrogen starvation. The iron superoxide dismutase and catalase that accumulated in the extracellular compartment were believed to minimize the cellular damage due to oxidative stress and to protect *Frankia* from toxic biotic or abiotic environments. The upregulation of a hemoglobin-like protein gene named *HbN*, and a gene encoding a raf kinase inhibitor protein homologous protein that regulates many signaling pathways was observed during N-starvation. However, since from the genome analysis of ACN14a, the full proteome database is expected to include about 3000 proteins, this analysis provided only partial information on the physiology of the actinobacterium. In one of the most recent publications, the transcriptome of ACN14a growing in N-fixing conditions was compared to that of the strain growing in ammonium-replete conditions, 252 genes were upregulated when the actinobacterial strain was starved of nitrogen, while 48 other genes were downregulated (Lurthy et al., 2018). The following sequences were found in the top ten upregulated genes: a gene coding for a putative transcriptional regulator, *nifD*, *glnII*, *nifH*, a sequence for a heat shock protein, *glnB1*, and a regulatory protein for nitrogen assimilation by glutamine

synthetase. Upregulation of accessory enzymes including amidases, proteases, nitrite reductase, and nitrite transporters was also observed. These enzymes could be involved in metabolic pathways that enable scavenging of nitrogen components and could play a role in the persistence of *Frankia* in soils (Lurthy et al., 2018).

Apart from searches for the transcriptional and translational modifications induced by nitrogen starvation in *Frankia* in the related literature, potential key molecular players involved in *Frankia* sporulation were also the object of searches. Two approaches were reported recently: (1) a comparison of protein contents in *Frankia* ACN14a spores and hyphae at different stages of the sporulation process; (2) comparative genomic analysis of *Frankia* proteomes with sporulating and non-sporulating actinobacteria (Bethencourt et al., 2019). The top five upregulated proteins include a putative phytase, a tyrosinase, a PadR family transcriptional regulator and an alkyldihydroxyacetonephosphate synthase (Bethencourt et al., 2019). However, whatever the studies, the link between the candidate genes identified in *Frankia* via transcriptome or proteome analyzes, and their effective roles, will have to be confirmed using functional analyzes when these tools are available for nitrogen-fixing actinobacteria.

29.3 *Frankia* as symbiont of actinorhizal plants

29.3.1 The infection processes

Like in legumes, *Frankia* can penetrate the plant root either intracellularly or intercellularly, depending on the host plant (Wall, 2000; Pawlowski and Demchenko, 2012; Santi et al., 2013). In *Hamamelidae* such as *Alnus*, *Myrica*, *Comptonia*, *Casuarina* and *Gymnostoma*, the infection process proceeds through root hair deformation and penetration. The process starts with root hair curling following the exchange of still unknown signals between *Frankia* and the host plant (Bogusz and Franche, 2015). After invagination of growing filaments of *Frankia* into the curled root hairs, infection proceeds intracellularly in the root cortex (Callaham et al., 1979; Berry et al., 1986). *Frankia* hyphae are surrounded by a plant-derived encapsulation layer enriched in polygalacturonans, in addition to host plasmalemma and cytoplasm. This interfacial matrix is the equivalent of the infection thread wall in legume nodules (Berg, 1999). Upon infection, cell divisions occur in the root cortex near the infected hairs, leading to a small external protuberance called a prenodule consisting of *Frankia*-infected and uninfected cells (Berry and Sunnel, 1990). Since the formation of the nodule primordium does not involve prenodule cells, it is hypothesized that the prenodule could be a primitive symbiotic organ (Laplaze et al., 2000). The nodule primordium results from mitotic activity in pericycle cells located opposite a protoxylem pole close to the prenodule (Duhoux et al., 1996). The infection of the developing nodule appears to be coordinated with the expansion of post-meristematic cells.

An intercolonization process can be observed in 17 genera distributed throughout the families *Eleagnaceae*, *Rhamnaceae*, *Rosaceae*, *Datisceae* and *Coriariaceae*, representing 70% of all actinorhizal species. In this infection pathway, root hairs are neither deformed nor invaded by the actinobacteria. *Frankia* enters the root by growing through the intercellular spaces between adjacent epidermal cells, penetrating the middle lamella, and progresses apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces (Valverde and Wall, 1999; Wall and Berry, 2008; Imanishi et al., 2015; Ibanez et al., 2016). Recent data obtained in the actinorhizal plant *Discaria trinervis* suggest that the host plant plays a major role in modifying both the size and composition of the intercellular apoplast in order to accommodate the filamentous microsymbiont (Fournier et al., 2018). Once the nodule primordium has developed from the pericycle, intracellular penetration by *Frankia* and the formation of infection threads is initiated acropetally in developing cortical cells of the nodule lobe primordium. No prenodule stage is observed in this infection process. Although the infection process has been studied in relatively few plant species, it has been suggested that the ancestral mode of infection is via intercellular penetration (Swenson and Benson, 2008; Ibanez et al., 2016).

In specific conditions, aerial nodulation has also been reported to occur on the trunks of actinorhizal trees of two *Casuarina* species, *C. glauca* and *C. cunninghamiana*. This stem nodulation requires high atmospheric humidity. Field experiments have shown that these nodules can fix nitrogen (Prin et al., 1991).

29.3.2 “Omics” of *Frankia* during the symbiotic process

Exploring the genomes of *Frankia* and comparing them to other nitrogen-fixing microorganisms indeed provided valuable preliminary information concerning the genes involved in nitrogen fixation and symbiotic interaction (Normand et al., 2007a; Tisa et al., 2016). A comparison of the proteome from free-living and symbiotic cells was performed on *Frankia* CcI3 by Mastrorunzio et al. (2009). Solute binding proteins were the most common class of secreted proteins observed in the symbiotic proteome, whereas hydrolytic enzymes were rarely detected. From these data, the authors

concluded that plant cell wall and/or membrane digestion was unlikely to be used by the actinobacteria during the colonization process.

The result of a transcriptome analysis was published in the *F. alni* ACN14a strain following the expression profiling of 6607 genes in free-living nitrogen conditions and symbiotic state (Alloisio et al., 2010). This enabled identification of a large number of upregulated symbiotic genes including genes related to nitrogen fixation, transcriptional regulation, signaling processes, protein drug export, protein secretion, liposaccharide, and peptidoglycan biosynthesis. Since strain ACN14a can establish a symbiotic relationship with plants of different actinorhizal families, the impact of the host plant on the transcriptome of the symbiont was then studied. Microarray analyzes of the transcriptomes in ACN14a from *A. glutinosa*, *A. nepalensis*, *Myrica gale* and *Morella rubra* did not reveal any significant differences. In a recent study, Lurthy et al. (2018), showed that the physiology of *in vitro* nitrogen fixation was markedly different when ACN14a fixed nitrogen in ammonium-free medium and when nitrogen fixation occurred under symbiotic conditions in nodules of *A. glutinosa*. Among 252 genes upregulated *in vitro*, 104 were also regulated in actinorhizal nodules, while 148 genes were not. These data led to the conclusion that molecular responses induced by nitrogen starvation differ in *Frankia* in free-living conditions and in symbiosis.

To identify the putative actinomycetal signals and key regulators involved in the dialog between *F. alni* ACN14a and its host *A. glutinosa*, an innovative study was recently performed by Pujic et al. (2019). A combination of proteomics and transcriptomics was used to investigate which genes were expressed and which proteins accumulated when *Frankia* hyphae ACN14a placed in dialysis tubes were exposed for 64 hrs to the root system of *A. glutinosa* plants starved of nitrogen. A total of 611 *Frankia* genes were upregulated and 460 were downregulated. The most upregulated genes were those encoding potassium transporter genes and the most down regulated genes were encoding a metallophosphoesterase and a fatty-acid-CoA ligase. Both downregulated and upregulated genes were dispersed over the *Frankia* genome. Together with this transcriptomic approach, a proteomic analysis was undertaken to determine the most abundant proteins. A total of 283 proteins were found to be more abundant after contact with root exudates and 160 less abundant. The most abundant proteins were labeled acyl-CoA dehydrogenases and a potassium transporter. However, at this stage of the analysis, it is still not possible to predict the biochemical structure of the signal(s) emitted by *Frankia* strains belonging to Cluster I (Pujic et al., 2019).

29.3.3 *Frankia* signaling molecules

Like in legumes, no single strain of *Frankia* can enter a symbiosis with all actinorhizal plants. The interaction is host specific and is based on a complex molecular dialog in the form of chemical exchanges and signals between the two partners. The infection process requires two major signaling events from both the host and the microsymbiont. Under limited nitrogen supply in the rhizosphere, the root system of the plant exudes flavonoid compounds that interact with *Frankia* and trigger the expression of genes coding for signaling molecules. In *C. glauca*, when an RNAi approach was used to downregulate the expression of the chalcone synthase gene (*CgCHS1*), a major reduction in nodulation was observed (Abdel-Lateif et al., 2013). The nodulation rate in the silenced plants was affected whereas the nodule structure was not. These data confirmed that flavonoids play a major role in the early stages of actinorhizal interactions.

With all the *Frankia* genomes currently available, it has been confirmed that the *Frankia* genomes characterized in clusters I and III do not contain the canonical *nodABC* genes that are necessary for the synthesis of the Nod factor, a lipochitooligosaccharidic signal that is essential for initiating symbiotic development in most legumes. It is also known that there is no symbiotic island, and that the identified nitrogen fixing (*nif*) genes are dispersed over the chromosomes (Normand et al., 2007a). Early characterization of *Frankia* deforming factor(s) resulting in root hair curling in *A. glutinosa* suggested that they were structurally divergent from the Nod factors of rhizobia (C  r  monie et al., 1999). In legumes Nod factors are heat stable, amphiphilic and chitinase sensitive (D  nari   et al., 1996; Fliegmann and Bono, 2015). *Frankia* factors from the symbiotic strain ACoN24d of *Alnus* were found to be heat stable, hydrophilic, and resistant to endochitinase and exochitinase from *Streptomyces griseus*. Moreover, neither the broad host range *Rhizobium* sp. NGR234 nor its purified factors elicited root hair deformation in *Alnus* and *Casuarina*, thus suggesting that actinorhizal plants do not recognize lipochitooligosaccharides. However, N-acetyl-glucosamine, the subunit of the Nod factor backbone, was detected in the *Alnus* root hair deforming fraction, leaving open the possible existence of a Nod-factor-related *Frankia* compound (C  r  monie et al., 1999).

The lack of a sensitive and reproducible bioassay to purify *Frankia* actinorhizal factor(s) involved in the intracellular infection process was one of the major obstacles to purifying the signaling molecules, until tools for the genetic transformation of the host plant *C. glauca* were developed (Smouni et al., 2002; Svistoonoff et al., 2010; Zhong et al., 2013; Froussart et al., 2016b). Gene constructs containing the reporter genes *gus* or *gfp* driven by plant promoters

expressed at specific stages of the symbiotic process (preinfection, root hair infection, prenodule and nodule) were introduced into *C. glauca* and the resulting transgenic plants were used to monitor the infection of the root system by the actinobacterium (Froussart et al., 2016a). Interestingly, the two promoters from early symbiotic plant genes, PCgCCaMK and PCgNIN, were found to drive gene expression during the preinfection stage, before the penetration of the *Frankia* hyphae into the curled root hairs (Svistoonoff et al., 2013; Clavijo et al., 2015). CCaMK is a calcium and calmodulin dependent kinase gene (Mitra et al., 2004; Lévy et al., 2004) and the Nodule INception (NIN) gene encodes a transcription factor involved in nodulation (Schauser et al., 1999; Marsh et al., 2007). Reporter gene expression driven by PCgCCaMK and PCgNIN was observed in developing root hairs from transgenic lateral roots of *C. glauca*, in response to *Frankia* CcI3 inoculation or to a supernatant of *Frankia*. This property, together with the possibility to elicit calcium spiking in host root hairs was used to obtain partial purification and characterization of the symbiotic factors secreted by *Frankia* CcI3 (Chabaud et al., 2016). The signal molecules were found to act at low concentrations, were hydrophilic, of low molecular weight and resistant to the degradation by a chitinase from *Streptomyces griseus*, thereby confirming the results published by Cérémonie et al. (1999) and indicating that the signaling molecules from *Frankia* in cluster I are chemically distinct from the chitin-based rhizobial and arbuscular mycorrhizal signaling molecules. The characterization of these molecules is not yet complete.

29.3.4 *nod* genes in *Frankia* strains in cluster II

Whereas it was not possible to identify *nodABC* genes in the first sequenced strains of *Frankia* belonging to clusters I and III (Normand et al., 2007a), a systematic search was indeed made for *nod* genes in all newly sequenced *Frankia* strains. In 2015, structural *nod* genes were identified for the first time in the uncultured *Frankia Datiscae* Dg1, the obligate symbiont originating from nodules of *Coriaria nepalensis* in Pakistan that has the ability to nodulate the non-perennial plant *D. glomerata* (Persson et al., 2015). The *nod* genes were arranged in two operons, and transcriptome analysis confirmed their expression in the nitrogen-fixing nodules. Furthermore, the possibility to partially complement a *nodC* mutant of *Rhizobium leguminosarum* by the homologous *nodC-Dg1* gene was shown; root hair deformation was observed in *Pisum sativum* following inoculation with the complemented rhizobial mutant, but nodulation was not restored. The authors concluded that either the transcription of *nodC-Dg1* was too low to provide enough Nod factor, or that the structure of the chitooligomer was partially affected.

In 2016, strain Dg2 originating from a *D. glomerata* plant growing in California was also sequenced (Van Nguyen et al., 2016). Like Dg1, this strain, which belongs to cluster II, contains the *nodABC* genes in a configuration that resembles that observed in Dg1. In addition, two copies of *nodH* coding for the sulfotransferase gene that regulates the specificity and the stability of the Nod factors in legumes were also found. *Nod* genes highly homologous to those found in the Dg1 genome were also observed in the genome of *Frankia* sp. NRRL B-16219 isolated from the soil rhizosphere of the actinorhizal plant *Ceanothus jepsonii* (Ktari et al., 2017a,b). The draft genome of NRRL B-16219 showed that the nodulation genes were clustered in two regions of the genome.

Since actinorhizal symbioses involving cluster II *Frankia* strains are thought to be the oldest actinorhizal symbioses, these data led to the hypothesis that canonical *nod* genes were probably lost during the evolution of *Frankia* symbionts. Interestingly, phylogenetic analyzes have shown that *Frankia* Dg1 Nod proteins are related to those of nodulator proteobacteria, suggesting a transfer took place between actinobacteria and proteobacteria during evolution (Persson et al., 2015).

29.3.5 Activation of the common signaling pathway by *Frankia*

Genome-wide transcription analyzes were first used in *C. glauca* and *A. glutinosa* to obtain an overview of the molecular mechanisms underlying the different stages of the actinorhizal plant response to *Frankia* infection (Hoche et al., 2011). Some data were then obtained with *D. glomerata* (Persson et al., 2015) and *Ceanothus thyrsiflorus* (Salgado et al., 2018). This knowledge, together with the development of techniques for the genetic transformation of a few actinorhizal plants (Smouni et al., 2002; Markmann et al., 2008; Svistoonoff et al., 2010; Froussart et al., 2016b) and the possible modification of gene expression of candidate genes from the host plants (Gherbi et al., 2008a,b), led to some major insights into the plant side during the actinorhizal nodulation process.

Based on the comparison with legume sequences, ESTs sharing significant homologies with genes from the common signaling pathway (CSP) were identified in *C. glauca* and *A. glutinosa* (Hoche et al., 2011). In legumes, this common signaling pathway (CSP) is necessary for both the nodulation by *Rhizobium* strains and the endomycorrhization process (Oldroyd et al., 2011). This pathway contains a receptor-like kinase SYMRK/DMI2, nuclear pores, and the potassium channel proteins required for the induction of calcium oscillations. A nuclear calcium- and calmodulin-dependent kinase

(CCaMK/DMI3) interacting with transcription factors (Cyclops/IPD3) is also part of this common pathway. The nuclear Ca^{2+} -spiking induced by AM fungi and nitrogen-fixing bacteria is decoded by the protein CCaMK, thus triggering infection and organogenesis programs. Expression analyzes performed in uninfected roots and nodules in *C. glauca*, *A. glutinosa* and the legume *Medicago truncatula* demonstrated that transcriptional regulation of the genes from the CSP in the two actinorhizal plants is comparable to that observed in legumes (Hocher et al., 2011).

To confirm these transcriptomic data, deep functional analyzes of *CgSYM/RK/DMI2* and *CgCCaMK/DMI3* isolated in *C. glauca* were then conducted following promoter analyzes, down-regulation in transgenic RNAi-plants (Gherbi et al., 2008b) and complementation of *L. japonicus* or *M. truncatula* mutants. Downregulation of *SYM/RK/DMI2* in *C. glauca* induced a severe reduction in nodulation by *Frankia* and endomycorrhization by the fungus *Rhizophagus irregularis*. Furthermore, *CgSYM/RK/DMI2* genes had the ability to complement knockout *L. japonicus symr*k mutants (Gherbi et al., 2008a). Concerning *CgCCaMK*, down-regulation of this gene resulted in reduced nodulation and AM fungal colonization in transgenic RNAi-plants (Svistoonoff et al., 2013). The function of *CgCCaMK* in endosymbioses was also supported by full complementation of the *M. truncatula dmi3* mutant for nodulation and colonization by AM fungi and by the observation of Ca^{2+} oscillations in *A. glutinosa* and *C. glauca* root hairs in response to *Frankia* exudate (Granqvist et al., 2015; Chabaud et al., 2016). Interestingly, when an auto-active form of *CgCCaMK* lacking the auto-inhibitory/CaM domain was expressed in *C. glauca* and *D. trinervis*, nodule organogenesis was observed in both plants in absence of *Frankia* (Svistoonoff et al., 2013). Since *D. trinervis* is an actinorhizal member of the Rosales infected by *Frankia* via the intercellular infection pathway, one can conclude that *CCaMKs* are central components of actinorhiza organogenesis, regardless of the mode of infection (Svistoonoff et al., 2013; Barker et al., 2017).

These results, together with transcriptomic data in *C. glauca* and *A. glutinosa* (Hocher et al., 2011) and *D. glomerata* (Demina et al., 2013), point to the existence of the CSP in actinorhizal plants, necessary for all root endosymbiosis involving *Frankia*, *Rhizobium* and arbuscular endomycorrhizal fungi.

29.3.6 Beyond the common signaling pathway

Transcriptomic data obtained in 2011 (Hocher et al.) provided some evidence that, in addition to the sequences belonging to the CSP, some candidate genes specifically expressed during the nodulation process were conserved between actinorhizal plants and legumes. NIN (for nodule inception) is a transcriptional regulator that is required for the formation of infection threads and for the initiation of nodule primordia in legumes. In contrast to CCaMK/DMI3, which is necessary for both nodulation and endomycorrhization, NIN specifically regulates the development of root nodules (Marsh et al., 2007; Vernié et al., 2015). In 2015, Clavijo et al. demonstrated that *CgNIN*, an actinorhizal *C. glauca* homolog to the legume *NIN* gene, plays a central role in the control of nodulation by *Frankia*. *CgNIN* expression was correlated with infection by *Frankia*, including during the preinfection stages in developing root hairs. Nodulation was impaired in knockdown RNAi-mutants and early root hair deformation responses were severely affected. Like in the *nin*-mutants in legumes, no mycorrhizal phenotype was observed and no induction of *CgNIN* expression was detected following inoculation of *C. glauca* by *R. irregularis*.

Using transcriptional and phylogenetic analyzes, Diédhiou et al. (2014) demonstrated that several other potential transcription factors homologous to those involved in legume–rhizobium and AM symbioses were also present in the two actinorhizal plants *C. glauca* and *A. glutinosa*. Therefore, although the canonical *nod* genes are not present in most *Frankia* strains, it seems that the genetic components of the symbiosis signaling pathway controlling actinorhizal and legume–rhizobia symbioses are strongly conserved and extend far beyond the CSP.

29.4 Contribution of *Frankia* to salt stress tolerance in actinorhizal plants

Salinity is one of the main environmental factors that limits crop productivity. It is encountered in all climates, but its effect is particularly dramatic in arid and semi arid regions. Salinity currently impacts more than 800 million hectares worldwide and affects an additional 0.3–1.5 million hectares of farmland every year thereby reducing crop yields.

It has been observed that some actinorhizal plants including those belonging to the Casuarinaceae family can tolerate a variety of abiotic stresses including salt stress (Mansour et al., 2016). Some species including *C. glauca* even grow better *in vitro* when their growth medium is supplemented with 50 mM of NaCl. Some studies have shown that the actinobacteria play a role in the ability of these plants to tolerate high concentrations of salt. Some *Frankia* strains including Allo2 and Cc16 can withstand concentrations of NaCl of up to 1 M whereas other salt-sensitive strains such as Cc13 cannot withstand more than 475 mM NaCl (Oshone et al., 2017). High salt concentrations were found to inhibit the initial steps of the interaction between *Frankia* and casuarinas trees, but when the trees were inoculated with a salt

tolerant *Frankia* strain before planting, they were able to tolerate NaCl concentrations > 500 mM, to develop nodules and fix nitrogen. When transgenic *C. glauca* trees expressing the GUS reporter gene under the control of promoters from early symbiotic genes were used, the signaling process preceding root hair infection was found to be deeply affected by high salt concentrations, whereas once the hyphae infected the lateral roots, plant genes from the common signaling pathway such as CCaMK were expressed at the expected levels.

Omics approaches were recently applied to three *Frankia* strains to identify genes that were upregulated under salt stress conditions (Oshone et al., 2017). Comparative analysis of the genomes from the two salt-tolerant strains CcI6 and Allo2, led to the identification of 153 single copy genes that were not found in the salt-sensitive strain CcI3. Genes with functions related to replication, recombination and repair, and cell wall/membrane biogenesis were identified. However, most specific genes found in salt-tolerant strains code for hypothetical proteins, suggesting novel mechanisms may be responsible for salt tolerance in *Frankia*. Analysis of transcriptomic data revealed that seven genes were responsive to salt, while proteome analysis revealed 19 osmotic stress-responsive proteins in CcI6. These preliminary data need to be confirmed by functional analyzes but suggest that the ability to maintain the integrity of the genetic material, the replication process, and the cell envelope are all important for salt tolerance in *Frankia* and that some hypothetical proteins could be involved in a novel mechanism for salt tolerance.

29.5 Conclusion

Although the microorganism *Frankia* remains difficult to study due to its slow growth rate, filamentous structure and cell heterogeneity, genome sequences and “omics” techniques have enabled valuable insights into these actinobacteria. The sequencing of more than 40 genomes confirmed that, with the exception of strains in cluster II, the canonical *nod* genes necessary for the nodulation process in legumes are not present in the genomes of *Frankia*, thus suggesting that a different signaling process underlies the actinorhizal symbiosis. Recent data obtained with the help of transgenic plants of *C. glauca* confirmed that the signaling molecules emitted by the symbiotic strain Cci3 are chemically different from the Nod factors secreted by most rhizobial strains during the interaction with legumes. Proteomics and transcriptomics have enabled major progress both in the identification of genes that are either overexpressed or repressed, and proteins that display differential accumulation in specific physiological conditions and during different stages of the symbiotic process. These data have led to different hypotheses concerning the key events of the symbiotic process. However, the development of genetic tools for the functional analysis of candidate genes in *Frankia* is still a major bottleneck. A few mutants have been reported in some *Frankia*, but the results remain limited to specific phenotypes that are easily selected. If confirmed, the recent claim concerning the possible introduction of a plasmid into *Frankia* by conjugation will open the way for functional analyzes of actinobacterial genes.

Many challenging questions remain to be answered. What are the signaling compounds produced by *Frankia* in clusters I and III? Are the signals for intracellular or intercellular infection processes the same? What is the basis of the specific recognition of the symbiont by the host? Which plant receptors are involved in recognizing these signals? Can knowledge of the actinorhizal symbiotic process help develop original strategies for the transfer of nitrogen fixation ability to cereals?

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Conflict of interest

The authors declare no conflict of interest.

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Advances in *Frankia* genome studies and molecular aspects of tolerance to environmental stresses

Nathalie Diagne^{1,2}, Pape Ibrahima Djighaly^{2,3,4}, Mariama Ngom^{2,3}, Céline Pesce⁵, Antony Champion⁶, Sergio Svistoonoff⁷, Valérie Hocher⁷ and Louis S. Tisa⁵

¹Institut Sénégalais de Recherches Agricoles/ Centre National de Recherches Agronomiques (ISRA/CNRA), Bambey, Senegal., ²LMI LAPSE, Centre de Recherche de Bel Air, Dakar, Senegal., ³LCM, IRD/ISRA, UCAD, Centre de Recherche de Bel Air, Dakar, Senegal., ⁴Département d'Agroforesterie, Université Assane Seck, Ziguinchor, Senegal., ⁵University of New Hampshire, Durham, NH, United States, ⁶DIADÉ UMR 232 IRD/CIRAD/University of Montpellier, Montpellier, France, ⁷LSTM, UMR 040 IRD/INRA/CIRAD, University of Montpellier/SUPAGRO, Montpellier, France.

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30.1 Introduction

Frankia strains are Gram +, aerobic, heterotrophic and filamentous bacteria found in association with plant root nodules or free-living in soil. The genus *Frankia* is a member of the *Frankiaceae* family and is the only genus in this family that is able to fix atmospheric nitrogen into biologically assumable form of nitrogen (Wall, 2000). *Frankia* are able to fix nitrogen under free-living conditions but also *in planta* through symbiotic root nodules (Simonet et al., 1990; Lechevalier, 1994). These bacteria are indeed able to develop a symbiotic association with a range of approximately 200 angiosperms, called actinorhizal plants, distributed among 25 genera and 8 plant families including *Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datisceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae* and *Rosaceae* (Benson and Silvester, 1993; Wall, 2000;). The *Frankia*-actinorhizal plant symbiosis plays important ecological and economic roles, such as soil stabilization, land reclamation, crop protection and timber and fuel wood production (Zhong et al., 2019). Under *in planta* conditions, *Frankia* converts atmospheric dinitrogen into ammonia (NH₃) which is assimilated by the plant. In return, actinorhizal plants supply the bacteria with carbon and energy sources. Excess nitrogen is excreted by some actinorhizal plants resulting in an improved soil fertility and *Frankia* is thus used as inoculum to improve plant performance in degraded environments. Zhong et al. (2019) observed that the tropical tree *Casuarina* spp., showed a higher tree survival and productivity on degraded sites following inoculation with *Frankia*. Isolation and culturing of *Frankia* strains is challenging because of their slow growth rate. The first pure culture of a *Frankia* strain was isolated from *Comptonia peregrina* nodules (Callaham et al., 1978). Since then, more than a hundred *Frankia* strains have been isolated from actinorhizal plants root nodule (Benson and Silvester, 1993). In pure culture, *Frankia* presents three major structures: vegetative hyphae (multiplication form), vesicles that are the site of nitrogen fixation under aerobic conditions, and complex sporangia called multilocular that constitute a dissemination and resistance form (Baker, 1990).

Even, if no *Frankia* strain specific to a single host plant species has been described yet (Pawlowski and Sprent, 2008), host specificity is present at different levels and a broad correspondence can be defined between the phylogenies of *Frankia* strains and actinorhizal plants. Based on molecular phylogenetic analysis, the genus *Frankia* forms four distinct clades or clusters (Normand et al., 1996; Benson and Clawson, 2000; Hahn, 2008). These clusters divide along plant host compatibility grouping. Furthermore, the recognition of different *Frankia* species delineation has just begun (Nouioui et al., 2016a,b; Nouioui et al., 2017a,b,c,d; Normand et al., 2018; Gtari et al., 2019). This speciation has been facilitated by the increase numbers of sequenced *Frankia* genome. To date, more than 38 *Frankia* genomes have been sequenced, annotated, and deposited in the NCBI database (Tisa et al., 2016). Some *Frankia* strains are resistant to abiotic stresses such as salinity, heavy metal, pH, and temperature (Faure-Raynaud et al., 1986; Moiroud, 1996; Diagne et al., 2013; Ngom et al., 2016a,b). *Frankia* genome sequencing has helped in the study of the molecular mechanisms involved in environmental stresses tolerance. This article reviews the current state of knowledge on *Frankia* genomic studies and on how it is used to decipher molecular mechanisms for stress adaptation.

30.2 Advances in *Frankia* genome studies

For the last years, genomic, transcriptomic and proteomic approaches in *Frankia* genome studies have been widely developed to better understand the actinorhizal symbiosis and *Frankia* responses to environmental stresses (Alloisio et al., 2010; Udway et al., 2011; Furnholm and Tisa, 2014; Rehan et al., 2014; Tisa et al., 2016; Furnholm et al., 2017; Oshone et al., 2017; Ghedira et al., 2018; Deicke et al., 2019; Gherbi et al., 2019; Rehan et al., 2019). Because of the absence of genetic tools for *Frankia* spp., these “-omics” studies were very valuable to provide molecular genetic approaches to address questions about the actinorhizal symbiosis and its microsymbiont. Just recently, a stable transformation was developed that allowed the introduction of plasmids into *Frankia* cells and functional expression of their genes (Pesce et al., 2019). This technique is the first step towards the development of genetic tools for *Frankia* and opens up the field of actinorhizal symbiosis to genetic approaches.

Historically, the first three *Frankia* genomes from three isolates belonging to clusters I and III of the four major lineages of the genus were sequenced in 2007 and compared to gain insight into the actinorhizal symbiosis determinants and the physiology of *Frankia* (Normand et al., 2007a; Normand et al., 2007b). The most noticeable difference among the three sequenced *Frankia* genomes was their sizes, ranging from 5.43 Mbp for the narrow host range *Casuarina* strain (*Frankia casuarinae* strain CcI3), to 7.50 Mbp for the medium host range *Alnus* strain (*Frankia alni* strain ACN14a), to 9.04 Mbp for the broad host range *Elaeagnus* strain (*Frankia* sp. Strain EAN1pec). Analysis of gene duplication, gene loss and gene acquisition through lateral transfer revealed that these three *Frankia* genomes are dynamic and have evolved as a function of their host specificity and biogeography (Normand et al., 2007b). Analysis of these three *Frankia* genomes revealed the presence of genes involved in nitrogen-fixing symbiosis (*nif*, *hup1*, *hup2* and *shc*), but the absence of *nod* genes suggesting the use of a novel nod-independent pathway for the infection process of actinorhizal plants.

Four major *Frankia* phylogenetic groups have been now sequenced, including members of noncultured cluster II (Ghodhbane-Gtari et al., 2013; Nouioui et al., 2013; Sen et al., 2013; Wall et al., 2013; Ghodhbane-Gtari et al., 2014; Hurst et al., 2014; Mansour et al., 2014; Tisa et al., 2015; Ngom et al., 2016a,b; Nouioui et al., 2016a; Oshone et al., 2016). Large scale comparative analysis of the sequenced genomes confirms the hypothesis that genome size was related to host specificity and biogeography ranges (Tisa et al., 2016; Normand et al., 2007b). Again, one of the most striking differences among the *Frankia* genomes is their size, which varied from about 5.4 Mbp for narrow plant host range strains (*Frankia* lineages Ic and II) to about 7.50 Mbp for medium plant host range strains (*Frankia* lineages Ia,b and III) to 9.0 and greater Mbp for broad host range strains (*Frankia* lineage III) and lineage IV strains that are unable to re-infect their host plants.

The presence of genes involved in nitrogen fixation (*nif*, *hup1*, *hup2* and *shc*) was confirmed for all of nitrogen-fixing *Frankia* strains. Curiously, genomes from *Frankia* lineage IV contained *hup1*, *hup2* and *shc* genes, but were totally devoid of any *nif* genes. However, these symbiotic genes were spread through the *Frankia* genomes and not clustered as in Rhizobia.

Genomes of the Cluster II lineage initially consisted of the uncultured *Frankia* symbionts of *Datisca glomerata* [Dg1 and Dg2] (Persson et al., 2011; Nguyen et al., 2016) revealed the presence of common *nod* genes as in the recently sequenced Cluster III *Frankia* strain NRRL B-16219 (Ktari et al., 2017a,b). However, the two Cluster II strains [*Frankia coriariae* BMG5.1 and BMG5.30] recently sequenced from *Frankia* do not have *nod* genes (Gtari et al., 2015; Gueddou et al., 2019). All together, these results suggest that two distinct nodulation pathways could be involved in actinorhizal symbioses. For the majority of *Frankia* species, the host plants would be infected through the nod-

independent pathway, while the use of a nod-dependent pathway may be involved in some hosts like *Ceanothus*. However, a symbiotic role of *Frankia nod* genes has not yet been clearly observed.

The availability of these numerous *Frankia* genomes allowed the recent development of other “omics” approaches such as transcriptomics (Alloisio et al., 2010; Popovici et al., 2010; Bickhart and Benson, 2011; Lee et al., 2013; Oshone et al., 2017; Lurthy et al., 2018) and proteomics (Alloisio et al., 2007; Mastronunzio et al., 2008; Mastronunzio and Benson, 2010; Udway et al., 2011; Ktari et al., 2017a,b; Oshone et al., 2017; Ghedira et al., 2018; Pujic et al., 2019; Béthencourt et al., 2019). The first transcriptomic work used whole-genome microarrays and aimed to characterize *Frankia alni* gene expression in symbiotic condition when hosted in *Alnus glutinosa* nodule (Alloisio et al., 2010). Since then, different transcriptome studies with DNA arrays or RNA seq approaches have been developed on different *Frankia* strains to reveal for example the set of genes expressed by these bacteria under in vitro nitrogen-replete or dinitrogen-fixing conditions (Bickhart and Benson, 2011; Lee et al., 2013; Lurthy et al., 2018). The first proteomic study based the *Frankia* genomes used two-dimensional gel separation, followed by MALDI-TOF analysis of individual protein spots from cultures grown with and without ammonium (Alloisio et al., 2007). Since then, different proteomic studies were developed with the aim to decipher the *Frankia*-actinorhizal symbiotic interaction. Mastronunzio and Benson (2010) integrated both proteomics data and *Frankia* genomic information to characterize the molecular basis of *Frankia*-actinorhizal symbiosis. Data were obtained from analysis of field-collected root nodules and *Frankia* spp. from the plant species, *Alnus incana*, and *Elaeagnus angustifolia*. For both *Frankia*-plant interactions, more than 1000 *Frankia*-proteins, were identified by annotation of proteins using the *Frankia* genome sequence and include proteins associated with signaling, motility, and detoxification of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Many of the high-abundance proteins were involved in energy and nitrogen metabolisms. Similar approaches were used to identify candidate proteins involved in the responsiveness of *Frankia* to plant phenolics (Popovici et al., 2010) and to desiccation (Ghedira et al., 2018). In a recent study, a next-generation proteomics approach was developed to uncover the key bacterial proteins involved in the early recognition of *F. coriariae* (Miotello et al., 2017). Several proteins with assigned and unassigned biochemical features were identified and a COG classification predicted that most secreted proteins were associated with metabolism of carbohydrates and amino acids and transport. All together, these studies led to a better understanding of the mechanisms of the establishment of actinorhizal symbioses.

The molecular responses to salinity of two *Frankia* isolates that have contrasting salt tolerance levels (salt-tolerant strain vs salt-sensitive strain) were also investigated through transcriptomics and proteomics analyzes (Oshone et al., 2017). Results of both approaches were convergent leading to the identification of several osmotic stress-responsive proteins, pointing thus some putative uncharacterized salt tolerance mechanisms (Oshone et al., 2017). Further investigations are now necessary to decipher the underlying salt tolerance mechanisms developed by *Frankia*. More recently, a next-generation proteomics approaches was developed in the alder microsymbiont *F. alni* under osmotic stress and a total of 2052 proteins were detected and quantified (Ghedira et al., 2018). These proteins included envelope-associated proteins that can help *Frankia* to grow under these stressful conditions.

30.3 *Frankia* tolerance to environmental stresses

Environmental stresses (salinity, heavy metal, temperature, and pH) have generally a negative impact on microorganisms living in the ecosystem. However, some microorganisms such as *Frankia* can adapt to these abiotic and biotic stresses. Interestingly, *Frankia* strains adapted to harsh environmental conditions improve host plants performance in these conditions.

30.3.1 Salinity tolerance

Salinity can negatively affect the structure and functioning of *Frankia* (Tani and Sasakawa, 2003a). Salinity tolerance levels for *Frankia* are strain-dependent. *Frankia* strain Ceql strain isolated from *C. equisetifolia* grows in the presence of 300 mM NaCl (Tani and Sasakawa, 2003b). A recent comparative study of multiple strains (Oshone et al., 2017) found that *F. casuarinae* strain Cc13 maintains a minimum inhibitory concentration (MIC) value of 475 mM NaCl while *F. casuarinae* strains Cc16 and Allo2 exhibited higher levels tolerance with a MIC value of 1000 mM NaCl. Several other *F. casuarinae* strains exhibited intermediate levels of NaCl tolerance with MIC values ranging 650–750 mM NaCl.

Salt stress also negatively affects *Frankia* nitrogenase activity (Oshone et al., 2013; Oshone et al., 2017). At 200 mM NaCl, nitrogenase activity is significantly reduced (15%). However some *Frankia* strains including strains Cc16 (Oshone et al., 2013) and CeD (Ngom et al., 2016a,b) are able to develop mechanisms to maintain nitrogen

metabolism activities under saline condition. Co-regulation of the nitrate uptake and the nitrogenase activity play a crucial role in governing the nitrogen status of bacteria strains during salt stress (Srivastava and Mishra, 2014). Under saline conditions, *Frankia* sp. strain Hsli0 showed a decrease in enzyme activities and better regulation of nitrogen metabolism, while the salt-sensitive *Frankia* sp. Hsli8 strain lacked the ability to regulate various steps of nitrogen metabolism under these conditions.

A genomic study revealed that the two salt-tolerant *F. casuarinae* strains CcI6 and Allo2 contained 153 shared single copy genes (most of which code for hypothetical proteins) that are not found in the salt-sensitive *F. casuarinae* strain CcI3 and moderately salt-tolerant *F. casuarinae* strain CeD (Oshone et al., 2017). RNA-seq analysis of one of the two salt-tolerant strains (*Frankia* sp. strain CcI6) revealed hundreds of genes differentially expressed under salt and/or osmotic stress. Among the shared single copy 153 genes, 7 genes are responsive to salt stress. One of these genes (*CcI6_RS22605*) was cloned into a vector and introduced into the salt-sensitive *F. casuarinae* strain CcI3. The expression of *CcI6_RS22605* gene in the transformant resulted in an increased salt tolerance (Pesce et al., 2019). All together, these results showed that salt tolerant *Frankia* strains develop several molecular mechanisms that are involved in salinity tolerance including cell envelope remodeling and transcriptional and translational regulation.

30.3.2 Heavy metal resistance

The most common heavy metals found at contaminated sites are arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn) (Diagne et al., 2013; Wang and Björn, 2014). Most *Frankia* strains are resistant to several heavy metals including AsO_4^{-3} , CrO_4^{-2} , SeO_2^{-2} , Pb^{+2} and Cu^{+2} (Richards et al., 2002; El Dein Abdel-Lateif et al., 2018). Both Pb^{+2} and Cu^{+2} resistances were suggested to result from a sequestration or binding mechanism. Although some prokaryotes have natural sequestering abilities (Gawali Ashruta et al., 2014), a mechanism studies of Pb^{+2} and Cu^{+2} resistance shows more specific mechanisms (Rehan et al., 2014; Furnholm et al., 2017)

Bioinformatics analysis of the *Frankia* genomes allowed prediction of several metal tolerance mechanisms that needed further experimental validation (Furnholm and Tisa, 2014). Four distinct patterns of metal homeostasis were identified: metal ion uptake transporters, efflux transporters, modification enzymes and both internal and surface binding proteins. Follow up “wet-lab” studies on Cu^{+2} , Pb^{+2} , and SeO_3^{-2} have supported these predicted mechanisms (Rehan et al., 2014; Furnholm et al., 2017; Rehan et al., 2019) and are described below.

Frankia inefficax strain EuI1c has an unusual but consistent pattern of Cu^{+2} resistance (Richards et al., 2002). Using a combination genome-guided and biochemical approaches, Cu^{+2} tolerance was further investigated to determine the mechanisms involved and identify potential genes involved (Rehan et al., 2014). Binding studies showed that Cu^{+2} was bound to the cells and unusual globular structures were observed on the cell surface. Scanning electron microscopy with energy-dispersive X-ray (SEM-EDAX) of the structures indicates that they were composed of copper and phosphate. Fourier transform infrared spectroscopy (FTIR) confirmed the presence of an increase in extracellular phosphate on the bacterial surface. The expression of five identified *cop* genes from bioinformatic work (Furnholm et al., 2017) increased in Cu^{+2} -stressed cells (Rehan et al., 2014). The working model is that copper is exported from the cells via CopA and CopCD transporter proteins and interacts with extracellular phosphate released by the bacteria. Thus, the Cu^{+2} resistance mechanism of *F. inefficax* strain EuI1c involves surface binding and transport proteins. Pb^{+2} tolerance by *Frankia* spp. was suggested to involve a binding or sequestration mechanisms (Richards et al., 2002). SEM, EDAX, and FTIR analyzes of Pb^{+2} -stressed cultures found that *Frankia* sp. strain EAN1pec undergoes membrane surface modifications and binds high quantities of Pb^{+2} (Furnholm et al., 2017). Proteomic analysis revealed Pb^{+2} -induced changes in exopolysaccharides, string response, the phosphate (*pho*) regulon and several transporter proteins. Two metal transporters (a Cu^{2+} -ATPase and cation diffusion facilitator), as well as several hypothetical transporters, were upregulated proteins and may be involved in metal export (Furnholm et al., 2017). The exported Pb^{+2} may be precipitated at the cell surface by an upregulated polyphosphate kinase. A complex model of the interplay of Pb^{+2} -upregulated proteins that contribute to the high level of Pb^{+2} resistance by *Frankia* sp. strain EAN1pec postulated.

Several *Frankia* strains have been shown to be resistant to elevated levels of selenite (Richards et al., 2002) and selenium resistance was predicted to involve alternative sulfate transporters and reduction of selenite to elemental selenium. Selenite-resistant *Frankia* strains reduce toxic, soluble, and colorless sodium selenite ($\text{Na}_2\text{SeO}_3^{-2}$) to nontoxic, insoluble and red colored elemental selenium (Se) (Richards et al., 2002; Rehan et al., 2019). *Frankia inefficax* strain EuI1c cultures exposed to 0.1 and 0.5 mM selenite completely reduced all of the selenite after 5 and 8 days (Rehan et al., 2019). SEM analysis of selenite-resistant *F. inefficax* strain EuI1c under selenite stress form nanosphere particles on the hyphal surface and inside the hyphae. EDAX analysis of the nanospheres confirmed that they are composed of

selenium and cell surface has changes in fatty acids, polysaccharides, carbohydrates and phosphate groups. Proteomic approaches also indicate transport protein involved in the mechanism. A mechanism of selenite reduction and nano-sphere transport through the membrane was suggested based on these results.

30.3.3 Temperature tolerance

Bacteria are able to grow across a wide range of temperatures, from very cold to very hot. Because of elevated temperatures in many areas, selection of temperature-tolerant *Frankia* strains is important choice in the preparation of inoculum to use under these conditions (Sayed et al., 1997). The influence of low temperatures on the growth and survival of *Frankia* strains (AgN21, AgN10ai, AgN12a and AgN15a) isolated in *Alnus* (Moiroud et al., 1984) showed that among the 3 of the 4 are unable to grow at temperatures $\leq 15^{\circ}\text{C}$, even after 10 weeks of incubation. However, *Frankia* strains resist to unfavorable temperatures, but are able to grow as soon as the temperature returned to 28°C for a few hours. These results were confirmed using *Frankia* strains AvcI1, CpI1 and LDAgp1 (Burggraaf and Shipton, 1982). For *Frankia* strains LDAgp1 and AvcI1, the optimum growth temperature is 30°C , while *Frankia torreyi* strain CpI1 had it at between 30°C and 35°C . The optimum growth temperature for *F. torreyi* strain CpI1 was confirmed and the values for *Frankia* strain EAN1pec, *Frankia* strain ACN1^{ag}, and *F. inefficax* strain EuI1c were $25\text{--}28^{\circ}\text{C}$, $30\text{--}33^{\circ}\text{C}$ and 30°C , respectively (Tisa et al., 1983).

Nitrogenase activity and vesicle production are also affected by temperature. *Frankia* strain ArI3 strain has an optimal temperature for nitrogenase induction from 25 to 28°C , but substantial activity was observed at 20 and 36°C (Murry et al., 1984). Vesicle formation and concomitant nitrogenase activity is affected by elevated temperatures in four *Frankia* strains (CpI1, ACN1^{ag}, EAN1pec and EUN1f) (Tisa and Ensign, 1987). For *F. torreyi* strain CpI1, no nitrogenase activity at 15°C and 36°C and optimum activity occurred at 25°C . *Frankia* strain ACN1^{ag} showed an optimum activity at 20°C and no activity at $30\text{--}36^{\circ}\text{C}$. *Frankia* strain EAN1pec show a tight window at 25°C and no nitrogenase activity at $30\text{--}36^{\circ}\text{C}$ with reduced activity at $15\text{--}20^{\circ}\text{C}$. *Frankia* strain EUN1f had nitrogenase activity over the $15\text{--}36^{\circ}\text{C}$ range, but optimum activity at 25°C . Vesicle production was inhibited at the restrictive temperatures.

Another biological activity affected by temperature is *Frankia* sporulation and spore germination. Sporulation by *F. torreyi* strain CpI1 is temperature-dependent and will only occur at temperatures below 33°C (Tisa et al., 1983). For *Frankia* strain DC12 and *F. inefficax* strain EuI1c, spore germination had an optimal temperature of 25°C and 30°C , respectively (Krumholz et al., 2003). Several isolates from *Casuarina* and *Allocasuarina* including *Frankia* strains CeI5, CgI1, CcI3 and AlI1, were shown to have spores germinate via growth on nutrient agar media (Tzean and Torrey, 1989). About 15% of the population of spores from *Frankia* strain CeI5 strain germinated within 2 days on a defined medium (BAP) at a temperature of $28\text{--}35^{\circ}\text{C}$. Under the same conditions, germination of *F. torreyi* strain CcI3 spores occurred in less than 0.5% of the population.

Extreme temperature exposure has a pronounced negative impact on bacterial metabolism. The molecular responses of *Frankia* spp. to temperature stress are yet not well studied. One hypothesis is that *Frankia* strains may have similar mechanisms to cold and heat shock effects. Analysis of the *Frankia* genomes reveals the presence of homologues for Cold Shock Proteins and Heat Shock Proteins found in other bacteria. Transcriptome analysis of salt and osmotic stress shows that some of the defense proteins are induced (Oshone et al., 2017). However, experiments of transcriptomes and proteomics are required to correlate these proteins to temperature responses.

30.3.4 pH tolerance

pH tolerance in *Frankia* is strain-dependent (Burggraaf and Shipton, 1982; Ngom et al., 2016a,b). For some *Frankia* strains, optimum growth occurred between pH 6–8 (Burggraaf and Shipton, 1982). Nevertheless, some *Frankia* strains are able to grow outside this range. For example, *Frankia coriariae* strain BMG5.1, a cluster II isolate, has an optimal growth pH of 9.5, the first alkalophilic *Frankia* strain (Gtari et al., 2015). A genome-guided approach was used to develop the methods of isolation of the first Cluster II isolate. Many other *Frankia* strains including LDAgp1, CcI3, AvcI1 and CpI1 grow optimally between 6.5 and 7.0. A study on twenty *Frankia* strains found that fourteen strains had no growth at pH 5 and only 3 strains are resistant to pH 4.6 for several weeks. None of these *Frankia* strain remained viable at pH 4.2 (Faure-Raynaud et al., 1986). Another study showed that seven *Frankia* isolates had significantly increased growth at pH 6.4, but only *F. torreyi* strain CcI3 is able to grow at pH 4.8 (Igual and Dawson, 1999). The survival of six *Frankia* strains isolated from a *Casuarina* plantation is often higher at pH 8, but not at a higher pH ≥ 8.5 (Mansour, 2003). These results help confirm that pH tolerance is strain-dependent in *Frankia*. Molecular mechanisms

for *Frankia* pH tolerance still remains unstudied. Comparative genomic analysis of the different *Frankia* strains could provide clues on the mechanisms of pH tolerance as will “omics” studies.

30.4 Conclusion

Among the beneficial microbes, *Frankia* plays an essential role for plant survival under harsh environment. The recent development of genomic resources for *Frankia* represent a major step that allowed to progress in the identification of molecular and protein key determinants to understand its adaptation to different environmental stresses such as salinity or heavy metal. However, further functional studies are now needed to understand their precise role. The recent development of a reliable methods allowing to manipulate the *Frankia* genes of interest (Pesce et al., 2019) represent an important step towards elucidating the elusive bacterial mechanisms involved in *Frankia* tolerance to environmental stresses.

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Chapter 31

Tripartite interactions between plants, *trichoderma* and the pathogenic fungi

Indu Kumari¹, Shikha Sharma², Geetika² and Mushtaq Ahmed²

¹Department of Zoology, Career Point University, Hamirpur, India, ²Department of Environmental Sciences, School of Earth and Environmental Sciences, Central University of Himachal Pradesh, Kangra, India

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31.1 Introduction

In modern agriculture, the role of beneficial fungi as biocontrol agents is of great importance due to their abilities to reduce the negative effects caused by various pathogens of the plant and animal origin. There are mainly three kinds of interactions between fungi and host plant that affect the plant growth and development i.e. neutral, negative and positive (Heil and Bostock, 2002). The fungi showing the neutral effect on the plants have negligible effect on plant growth and development. While negative and positive interactions do have a significant impact on plant growth and development (Choudhary et al., 2007). During negative interactions, the fungi have an adverse effect on the plants and such fungi are known as pathogenic fungi. There is the number of fungi in the fungal kingdom that causes serious plant diseases. These pathogenic fungi mainly include species of *Fusarium*, *Pythium*, *Aspergillus*, *Botrytis*, *Puccinia*, *Rhizoctonia* and *Alternaria* (Benítez et al., 2004). All these pathogenic fungi are able to affect a number of economically important plants by causing diseases in the host plants (Ma et al., 2013; Singh et al., 2018). These pathogenic fungi affect the host plant directly by physical contact or indirectly by the production of toxins (Jestoi, 2008). The basic impact of these fungi is to weaken the host defense system to meet their own requirements (Benítez et al., 2004). Once the pathogenic fungi are able to establish inside the host plant their negative effects are manifested as plant diseases (Harman et al., 2004). When phytotoxins are produced inside the host plants, the infected host plants show retarded growth, damping off seedlings, delayed or no seed germination and/or leading to plant death (Harman et al., 2004). *Fusarium* spp. alone are able to cause various serious diseases like wilts, blights, rots, and cankers (Ma et al., 2013). In order to cause disease in the host plants, various factors such as environmental factors (presence or absence of other microbes), physical factors (temperature, light, etc.) and chemical factors (pH, carbon, nitrogen, water, etc.) play an important role (Netzker et al., 2015). These factors are responsible for the pathogen survival, spore germination, hyphal growth, host tissue penetration and colonization in the host plant (Van Loon and Bakker, 2005). If the suitable conditions exist for pathogenic fungi they are able to infect the host plant (Bae et al., 2006). Generally, the host plant root tips are very susceptible for the entry of the pathogens. For example, *F. oxysporum* produces enzymes cellulases and glucanases that weaken the cell wall of the host plant root tips and establish a connection for hyphal entry (Bae et al., 2006). Once *F. oxysporum* enters through root tips it moves further through the vascular system and causes disease in the host plant (Bae et al., 2006). Most pathogenic fungi are host specific in their nature but some pathogenic fungi such as *F. proliferatum* are able to infect a wide host range under various climatic and geographical regions over the globe (Stępień et al., 2011).

In some cases, these pathogenic fungi can also survive as endophytes within the host plant without showing any visible disease symptoms but when these infected plants are consumed, they show their toxic effects on the consumer, for instance, *F. proliferatum* and *F. moniliforme* (Bacon and Nelson, 1994). In nutshell, whenever there is a successful interaction between the pathogenic fungi and the host plant, the pathogenic fungi will impart the damaging effect on its host growth and development. Therefore, the plant growth promoting fungi (PGPF) such as *Trichoderma* spp. have been exploited for ameliorating the negative effects of the pathogenic fungi (Benítez et al., 2004; Hidangmayum and Dwivedi, 2018). Further, it can be better used sustainably in the agricultural field to enhance plant growth as well as yield (Benítez et al., 2004; Hidangmayum and Dwivedi, 2018). Also, biofertilizers and biopesticides are the only alternative to harmful chemical pesticides and fertilizers that are used to increase crop yield (Ahmed and Upadhyay, 2009). Plant growth promoting microbes like species of *Pseudomonas* and *Trichoderma* is being successfully used for plant disease management and increasing crop yield (Ahmed and Upadhyay, 2009; Sharma et al., 2017b). The microbes associated with the plants are generally free-living and helpful to the plants (Harman, 2006). Fungi such as *Aspergillus*, *Rhizoctonia*, *Penicillium*, *Piriformospora*, *Phoma*, and *Trichoderma* have been reported enormous mechanisms to help plants in biotic and abiotic stress management (Harman, 2006).

Most successful biocontrol agents in today's agriculture are the species of *Trichoderma* as around 60% of the bio-fungicides which are used in agriculture are *Trichoderma*-based formulations (Verma et al., 2007). *Trichoderma* as bio-control agent is of great interest because of its high reproductive capacity, mycoparasitic activity, secondary metabolite production ability and growth inhibition activity of plant pathogenic fungi (Benítez et al., 2004; Harman, 2006) (Fig. 31.1). *Trichoderma* are ubiquitous, avirulent, opportunistic plant symbionts and can also act as parasites for another group of fungi. The endophytic fungus *Trichoderma* colonizes the root surfaces and penetrates deep into the root epidermis (Harman et al., 2004). The biological control abilities have been shown in the different species of *Trichoderma* including *T. harzianum*, *T. viride*, *T. reesei*, *T. virens* and *T. asperellum* (Singh et al., 2018). Species of *Trichoderma* such as *T. harzianum* and *T. atroviride* showed multiple interactions with the crop plants as well as with fungal pathogens (Woo et al., 2006). *Trichoderma* interacts with many plant species by forming mutualistic endophytic relationships and secretes a variety of compounds that are responsible for localized or systemic resistance in plants. Inoculation of the plants with *Trichoderma* spp. combats the diseases caused by pathogenic fungi along with plant nutrient management (I Chet et al., 1997). *T. harzianum* is most active agent present in recently used biofertilizers and bio-pesticides. *Trichoderma* spp. antagonize wide spectrum of phytopathogenic fungi by a variety of mechanisms including colonization, competition, mycoparasitism, antibiosis and induced resistance (Howell, 2003; Vinale et al., 2006). Among all the mechanisms mycoparasitism and induction of plant defense are the most important biological control mechanisms by *Trichoderma* spp (Mukherjee et al., 2012). Colonization of the plant roots by *Trichoderma* strains bring about increased levels of different enzymes like β -peroxidases, chitinases, defense-related enzymes, 1,3-glucanases and hydroperoxidase lyase of lipoxygenase–pathway (Harman et al., 2004). *Trichoderma* spp. also, play a significant role in the process of bioremediation of soil (Ranasingh et al., 2006). The fungi are able to degrade a range of insecticides

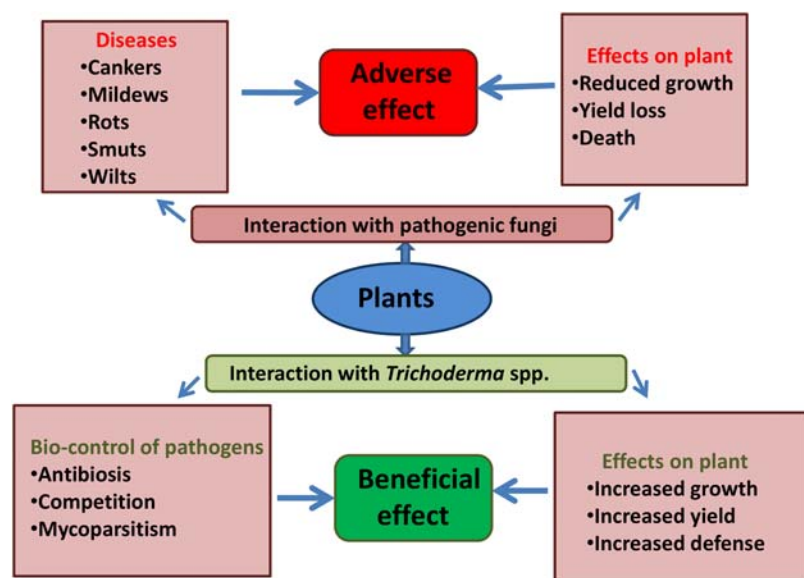


FIGURE 31.1 Effect of pathogenic fungi and *Trichoderma* Spp. On the host plant. *Trichoderma* is present at the interface of the plant host and the pathogenic fungi. It enhances the plant growth by secretion of Phytohormones; strengthen the defense strategies of the plant and nutrient availability. *Trichoderma* suppresses the growth of the pathogenic fungi by Mycoparasitic activity, competing for nutrients and production of antibiotic/secondary metabolites.

like organophosphates, organochlorines, and carbonates successfully (Ranasingh et al., 2006). Plant growth promoting microorganisms are important in terms of environmental sustainability, but more work is needed to explore their interactions at the microniche of the host plant to manage agroecosystems in a sustainable way. This chapter aims to highlight the biocontrol abilities of *Trichoderma* spp., their role in plant growth promotion and their effect on plant development and the tripartite interactions operating among *Trichoderma*, the host plant and the pathogenic fungi.

31.2 *Trichoderma* as biofertilizer and biocontrol agent

The chemical pesticides, growth regulators and inorganic fertilizers are used abundantly in modern agricultural practices. These chemicals raised crop yields as well as environmental pollution, loss of crop diversity and resulted in deleterious effects on living beings (Kumar and Ashraf, 2017). Plant-growth promoting microbes (bacteria and fungi) showed potential as biofertilizers and biocontrol agents (BCAs) in agriculture so that the reliance on synthetic agrochemicals can be reduced (Mishra et al., 2017). These microbes can also be used in the bioremediation processes through the removal of unmanageable compounds from the ecosystem, help in combating climate changes, and also relieve abiotic stress (Mishra et al., 2017). Among fungal BCAs, *Trichoderma* spp. are most commonly used and are also being used as effective biofertilizers. Different species of *Trichoderma* are used as biofertilizers and their application in the fields resulted in plant growth promotion, enhanced crop yield, and increased nutrient availability. Effective biofertilizers like *Trichoderma* spp. reduce the chemical burden in soil and environmental pollution caused by these chemicals (Molla et al., 2012; Kumar and Ashraf, 2017). Whole genome sequence of 17 different species of *Trichoderma* genus has been sequenced till now. These are *T. reesei*, *T. virens*, *T. longibrachiatum*, *T. atroviride*, *T. asperellum*, *T. hamatum*, *T. harzianum*, *T. citrinoviride*, *T. brevicompactum*, *T. gamsii*, *T. atrobrunneum*, *T. arundinaceum*, *T. koningiopsis*, *T. guizhouense*, *T. koningii*, *T. pleuroti* and *T. parareesei* (<https://www.ncbi.nlm.nih.gov/genome>). The whole-genome sequence analysis of promising BCA, *T. gamsii* T6085 strain has shown its growth inhibition ability against *Fusarium* head light disease along with the reduction in mycotoxin accumulation in the host plant (Baroncelli et al., 2016). *T. hamatum*, another important biocontrol agent from genus *Trichoderma* has been sequenced and compared with other species of *Trichoderma* genomes (*T. atroviride*, *T. virens*, and *T. reesei*). The genome of *T. hamatum* GD12 strain has been sequenced which showed its uniqueness in plant growth promotion, exhibit mycoparasitism against *Sclerotinia sclerotiorum* (pathogen of agronomic crops), biocontrol activity against the soil pathogens and induction of systemic resistance in a range of plants (Studholme et al., 2013). Genome analysis of different *Trichoderma* species helped in the exploitation of beneficial traits of these fungi for sustainable agricultural practices (Kumar and Ashraf, 2017). Plant diseases involve interactions among the plant pathogens and the environment whereas; BCAs manage the diseases by interacting with these components. For these successful interactions for controlling plant diseases, *Trichoderma* exhibits several mechanisms like mycoparasitism, competition, induction of plant defense, plant growth promotion and plant root colonization (Kumar and Ashraf, 2017). Mycoparasitism is the direct effect (parasitism) of one fungus on the other fungus (Weindling, 1932). *Trichoderma* spp. show parasitism against a variety of other fungi, the first report on the antagonistic activity of *T. lignorum* against *Rhizoctonia solani* was given in 1932 by Weindling (Weindling, 1932). In mycoparasitism different events takes place starting from the chemotropism and detection of other fungi, attachment and coiling around the fungi, penetration of the fungal cell wall and finally host cell digestion (Chet et al., 1981). Another mechanism involves competition for nutrients and space between the pathogenic fungi and BCA to better adjust them in the environment. The most common cause of death of the microorganisms is starvation and competition for limiting nutrients in their immediate environment (Chet et al., 1997). Competition occurs for micronutrients like iron which is important for the viability of most filamentous fungi. During iron deficient conditions biocontrol agents like *Trichoderma* spp. synthesize siderophores (low-molecular-weight iron-chelators) to overcome iron scarcity (Benítez et al., 2004; Eisendle et al., 2004). Siderophores chelate iron, form the siderophores-Fe complex and thus making it accessible for BCAs (Mukhopadhyay and Mukherjee, 1998; Benítez et al., 2004). *Trichoderma* spp. are highly specialized in taking up of soil nutrients in comparison to the pathogens by siderophores formation so that iron remains available in lower quantities (Mukhopadhyay and Mukherjee, 1998; Benítez et al., 2004). However, there is need to carry out analysis about the proteins involved in the transport of siderophores. Like other mycorrhizal fungi, *Trichoderma* stains also colonize plant roots and secrete a variety of compounds beneficial in plant growth and defense mechanisms (Harman et al., 2004). Addition of *Trichoderma* strains into rhizosphere soil of the plants protects them against numerous plant pathogens (viral, bacterial and fungal) through the induction of induced systemic resistance (ISR), systemic acquired resistance (SAR) and hypersensitive response (HR) in the plants (Benítez et al., 2004; Harman et al., 2004). The increase in metabolite concentration and defense related enzymes were seen as a result of resistance (Benítez et al., 2004). Chalcone synthase and phenyl-alanine ammonia-lyase (PAL) are the enzymes

involved in the defensive mechanisms through the biosynthesis of chitinases, glucanases and phytoalexins (HR response) (Benítez et al., 2004). The experiment conducted on cucumber plant showed that two genes encoding for phenylalanine, and hydroperoxide lyase present in *T. asperellum* are involved in ISR and phytoalexin accumulation for resistance against *Pseudomonas syringae* (Yedidia et al., 2003). Another important mechanism is antibiosis which occurs due to interactions of antibiotics (low-molecular weight compounds) synthesized by *Trichoderma* strains which antagonize the growth of the pathogenic fungi (Vey et al., 2001). The volatile or non-volatile compounds produced by *Trichoderma* spp. are peptaibols, antibiotics, harzianic acid, tricholin, alamethicins, gliovirin, viridian, glisoprenins, massoialactone, heptelidic acid and others (Vey et al., 2001). The biocontrol activity of the *Trichoderma* spp. and their mechanism is well studied by characterization of the genes present in them. Genomic studies revealed the presence of differentially expressed genes (biocontrol genes) in *Trichoderma* spp. which allows the use of fungi as BCAs, plant growth promotional activities and abiotic resistance (Sharma et al., 2011; Singh et al., 2018). Variety of biocontrol proteins has been isolated, cloned and characterized. There is the number of cellular factors that are involved in mycoparasitism of *Trichoderma* spp. such as Cell Wall Degrading Enzymes (CWDEs) (chitinases, glucanases, galacturonases, xylanases and proteases), hydrolytic enzymes, cytoskeletal elements, stress tolerant genes, and cell adhesion protein (Sharma et al., 2011). Stress tolerant genes (e.g., *pacC* gene involved in sensing pH variation response) help BCA to survive and thrive even under unfavorable conditions (Benítez et al., 2004). Cell adhesion proteins are involved in the recognition of host fungi by recognizing the carbohydrates attached to lectins on the cell wall of the host fungi (Harman et al., 2004). Chitinases (e.g. *chit 102* and *chit42*), glucanases (e.g. 1,3- β -glucanases) and proteases (e.g. alkaline proteases) enzymes are involved in the dissolution of a cell wall of the host fungi (Inbar and Chet, 1995; Harman et al., 2004). Hydrolytic enzymes (e.g. monooxygenases) also enhance the biocontrol potential of *Trichoderma* spp. by accelerating mycoparasitism process (Harman et al., 2004). Cytoskeletal elements such as tubulins are also supposed to help in coordination of CWDEs within the host fungi (Inbar and Chet, 1995).

31.3 The activity of *Trichoderma* in plants

Trichoderma spp. control plant pathogens, increase plant growth and evoke plant defense mechanisms. Colonization of plant roots by the fungus provides protection against pathogenic microorganisms in the inhabited zone as well as robust root system development for the improvement in nutrient and water uptake capacity (Contreras-Cornejo et al., 2016). The treatment of soil/seed with *Trichoderma* increases the percentage of seed germination, either by activation of enzymes and phytohormones or by increasing nutrient availability in soil and changing soil microflora (Bezuidenhout et al., 2012). Its application improves the active-uptake mechanism of plants by increasing the uptake of a variety of nutrients like phosphorous, iron, manganese, sodium and copper by the plants (Harman et al., 2004). The fungus increased root colonization, resulted in intensified levels of defense related enzymes which include chitinases, 1,3-glucanases, β -peroxidases and hydroxide lyase of lipoxygenase-pathway (Harman et al., 2004). In soybean cells, secondary metabolites produced by fungus induces variations in intracellular levels of Ca^{2+} (Navazio et al., 2007). *Trichoderma* mediated plant immunity is also reported in cotton, rice and *Arabidopsis thaliana* through reactive oxygen species (ROS) like hydrogen peroxide and nitric oxide (Contreras-Cornejo et al., 2016). Mitogen-activated protein (MAP) kinases involved in the plant defense signaling were also activated during inoculation of plant roots by *T. asperellum* in cucumber (Shoresh et al., 2005). The increase in MAP kinase activity is obligatory for resisting the attack of the pathogens (Contreras-Cornejo et al., 2016). *T. asperellum* T203 is known to modulate the expression of *LOX1* (Lipoxygenase 1) and *PAL1* (phenylalanine ammonia lyase) genes of jasmonic acid (JA) and salicylic acid (SA) biosynthetic pathway (Shoresh et al., 2005). The fungus also controls the components of ethylene signaling, *ETR1* (ethylene receptor 1) and *CTR1* (constitutive triple response 1) (Shoresh et al., 2005). The main signal transduction pathways are JA, SA and ethylene involved in *Trichoderma* mediated ISR which sensitizes the host plant to respond to the pathogenic attack more efficiently (Shoresh et al., 2005). *Trichoderma* spp. are producers of different secondary metabolites like peptaibols, volatile/non-volatile terpenes, siderophores, and pyrones, etc. The metabolites identified from the fungus were reported to perform specialized functions as mediators in chemical communication (Contreras-Cornejo et al., 2016). Secondary metabolites like konigin A produced by *T. koningii* and 6-pentyl- α -tyrone produced by *T. harzianum* also act as plant growth regulators (Cutler et al., 1986). Gliotoxin, a metabolite produced by *T. harzianum* mimics the action of gibberellic acid which is a plant growth hormone and is involved in the process of seed germination (Bezuidenhout et al., 2012). In initial stages of plant-microbe interactions, some proteinaceous compounds and auxin-like metabolites are released by *Trichoderma* that are recognized by the plant roots that alter hormonal mechanisms which control growth and development of the plants (Contreras-Cornejo et al., 2016). Stimulation of the plant growth by microorganisms is also dependent on the production of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase (ACCD) involved in the

cleavage of ACC (precursor of ethylene) (Contreras-Cornejo et al., 2016). *T. asperellum* has gene encoding ACCD responsible for the elongation of roots in the rape plant (Viterbo et al., 2010; Contreras-Cornejo et al., 2016). Reduced ethylene levels in the plant due to decrease in its precursor (ACC) by ACCD produced by *Trichoderma* spp. is responsible for the enhanced growth of the plant (Viterbo et al., 2010).

Studies showed that in the greenhouse and the field conditions, *Trichoderma* spp. suppress the plant diseases and growth of the pathogenic fungi that are in contact with the plant tissues (Harman et al., 2004; Contreras-Cornejo et al., 2016). Volatile organic compounds (VOCs) released by *T. virens* play an important role in the protection of *A. thaliana* against *Bacillus cinerea*, a plant pathogen causing necrosis and death of infected seedlings (Contreras-Cornejo et al., 2014b). The interactions of the plant roots with *Trichoderma* also help them to resist abiotic stress and induce the growth of the plant (Björkman et al., 1998). Underwater deficit conditions *T. harzianum* helps tomato plants by modulating the expression of genes that encode enzymes like superoxide dismutase, ascorbate peroxidase, and catalase, the scavengers of ROS in root and shoot of the plants. The enzymes help the plants to respond less to water deficit conditions and help in their growth (Björkman et al., 1998). Under another abiotic stress such as higher salinity conditions the growth and development of *A. thaliana* plant was also studied in the presence and absence of *Trichoderma* (Contreras-Cornejo et al., 2014a). In the absence of *Trichoderma*, higher salinity affected primary root length, lateral root development, and root hair induction adversely. Shoot diameter decreases with decrease in total chlorophyll content of the plant. In contrast, *Trichoderma* inoculation resulted in greater tolerance to saline stress by accumulation of abscisic acid, ascorbic acid (antioxidant) and L-proline (osmolite) (Contreras-Cornejo et al., 2014a).

Plant growth significantly increases in the presence of *Trichoderma* isolates and one reason for this increase is solubilization of natural phosphates by the fungus (Borges Chagas et al., 2015). Studies on phosphate solubilization by *Trichoderma* spp. revealed high potential of these biofertilizers to solubilize it. Solubilization starting on the third day of *Trichoderma* inoculation and an increase in the growth of rice plants was seen in comparison to the control (Borges Chagas et al., 2015). It has also been suggested that phosphate solubilization by microorganisms is due to decrease in pH levels of the culture medium (Schoebitz et al., 2013; Borges Chagas et al., 2015). *T. harzianum* strain T24 was also studied for its role in nitrogen fixation, hydrolytic enzyme production and growth promotion of the tomato seedlings (El-Katatny, 2010). A synergistic effect of biocontrol fungus *T. harzianum* with rhizobacterium *Azospirillum brasilense* on nitrogen fixation was demonstrated (El-Katatny, 2010). Stimulation of nitrogen fixation and increase in nitrogenase activity of bacteria was seen during the mutual growth of fungus and rhizobacterium. Inoculation of tomato seedlings with *T. harzianum* strain positively affects its growth rate and nitrogen fixation tendency of bacteria (El-Katatny, 2010).

31.4 Effect of fungi on the plant growth and development

Trichoderma spp. have been reported to increase plant growth parameters, photosynthetic activity, nutrient absorption capacity, tolerance to biotic and abiotic stress and induction of defense mechanisms in the host plants (Shoreesh et al., 2010). Soybean plants when inoculated with *T. harzianum*, showed 125% increase in plant yield (Harman, 2000). Likewise when dwarf tomato plants were subjected to *T. viride* treatment, there was 28% increase in plant height and 8% increase in plant weight (Contreras-Cornejo et al., 2016). Similarly, during greenhouse trials when cabbage seedlings were treated with *T. longipile* and *T. tomentosum*, there was 58%–71% increase in the leaf surface area, 91%–102% increase in dry shoot weight and 100%–158% increase in dry root weight (Rabeendran et al., 2000). The secretion of secondary metabolites by *Trichoderma* spp. in the plant rhizosphere lead to the beneficial effect on the host plant (Zeilinger et al., 2016). Some of these secondary metabolites have also ability to suppress pathogens growth along with plant growth promotion activity (V. Sharma et al., 2017a). Various secondary metabolites such as koniginins, trichocaranes, pentyl- α -pyrone, harzianolide and harzianic acids are some of the compounds that have been reported to exhibit plant growth promoting activity (Francesco Vinale et al., 2014). Along with these secondary metabolites some auxin like hormones produced by various *Trichoderma* spp. are also helpful in increasing the plant growth (Contreras-Cornejo et al., 2009; Contreras-Cornejo et al., 2015; Contreras-Cornejo et al., 2015). Generally, plant roots release some exudates in the rhizosphere such as organic acids, sugars, lipids and amino acids (Contreras-Cornejo et al., 2016). These exudates attract *Trichoderma* towards the host plant roots. *Trichoderma* colonizes the roots and increases lateral root development, root hair growth and mineral absorption in the plant (Contreras-Cornejo et al., 2016). When various secondary metabolites and auxin, jasmonic acids and salicylic acids like hormones are released by *Trichoderma* spp., it results in increased photosynthetic activity, defense response and ultimately plant growth and development (Contreras-Cornejo et al., 2016).

31.5 Induction of systemic resistance

Whenever a plant is attacked by any pathogen, it activates defense response against the pathogen (Contreras-Cornejo et al., 2016). For their own protection, the plants have various defense mechanisms against the pathogens (Ryals et al., 1996). The pathogens that cause tissue necrosis, activate systemic acquired resistance (SAR) in the plants (Ryals et al., 1996). Likewise, when non-pathogenic microorganisms colonize the plant roots, they activate induced systemic resistance (ISR) in the host plant (Van Loon et al., 1998; Conrath, 2011) (Fig. 31.2). Both of these mechanisms i.e. SAR and ISR are capable of restricting pathogen growth and suppression of disease in the infected plant (Conrath, 2011). Activation of SAR requires endogenous accumulation of salicylic acid (SA), whereas, activation of ISR does not require SA accumulation (Pieterse et al., 2009). ISR requires continuous responsiveness to the plant hormone jasmonic acid (JA) and ethylene (Pieterse et al., 2009). Various plants have ability to detect conserved motifs or domains of microbes called microbe-associated molecular proteins (MAMPs) and pathogen-associated molecular proteins (PAMPs) (Hermosa et al., 2012). This molecular recognition of MAMPs and PAMPs by the host plant is also responsible for the activation of SAR and ISR (Harman, 2011). Both of these above mentioned defense mechanisms are very important for the plants to fight against a number of pathogens. *Trichoderma* enhanced the plant defense under low nitrogen mediated conditions by changing nitric oxide, ROS production, nitrate transporter genes induction, production of SA and induction of defense, SAR marker genes like Non-expressor of Pathogenesis-related gene 1 and pathogen-related proteins (Hermosa et al., 2011). *Trichoderma* spp. are able to produce MAMPs and hence initiate signaling cascade (SA, JA and/or ethylene) within the host plant (Hermosa et al., 2011). For example, *T. asperellum* is able to produce ISR both SA and JA/ethylene signaling in *Arabidopsis* while colonizing the plant roots (Yoshioka et al., 2012). The secondary metabolites produced by *Trichoderma* spp. like trichothecenes act as MAMPs and induce cell signaling that stimulates defense system of the plants (Kumari et al., 2016). *Trichoderma* spp. are also capable of activation and accumulation of defense related compounds such as polyphenols, terpenoids, a phytoalexin, ascorbic acid, and glutathione, etc. within the host plant (Howell et al., 2000). MAPK (Mitogen-activated protein kinases) pathway generally transport information signal from sensors to produce cellular response against the pathogens (Shoresh et al., 2006). It has been reported that *T. asperellum* activated MAPK and production of phenolic secondary metabolites in cucumber that provided protection against *Pseudomonas syringae* pv. *lachrymans* to the plant (Iris Yedidia et al., 2003). *T. virens* and *T. atroviride* are reported to increase the level of phytoalexin in *Arabidopsis* and hence help in triggering induced defense mechanism (Contreras-Cornejo et al., 2011). *T. asperelloids* induces a rapid increase in expression of WRKY18, WRKY33, WRKY40 and WRKY60 transcription factors (Brotman et al., 2013). These transcription factors, in turn, activate JA pathway response (Brotman et al., 2013). Moreover, *Trichoderma* spp. are reported to induce systemic resistance in the number of crop plants such as wheat, rice and maize (Singh et al., 2018). Thus apart from triggering defense system in the host plant *Trichoderma* spp. also, increase plant yield (Singh et al., 2018).

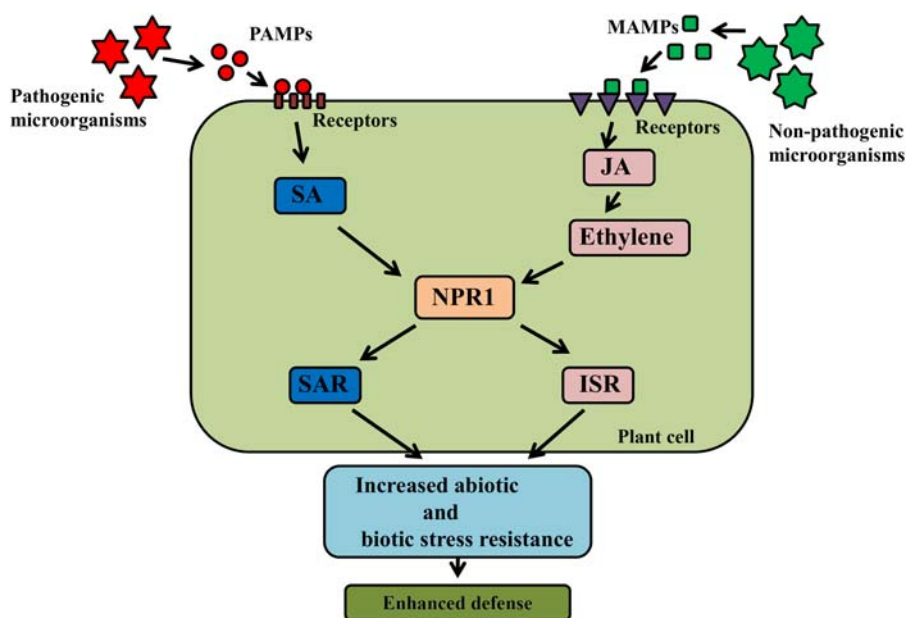


FIGURE 31.2 Overview of a plant defense mechanism. Non-expressor of pathogenesis-related gene 1 (NPR1) is a protein analogous to Ankyrin family proteins. This protein resembles the IF- κ B inhibitor, which participates in animal innate immunity. This protein is involved in SAR, ISR and also in resistance gene-dependent resistance and basic resistance. Secondary metabolites secreted by *Trichoderma* act as MAMPs that lead signaling in the plant which induces defense system of the plant. However, molecules from the pathogenic fungi act as PAMPs.

31.6 Changes in metabolome of host plant, PGPF and pathogenic fungi

The interactions among various microorganisms and the plants lead to a change in the metabolic profile of all interacting species due to their presence or absence around their neighborhood (Contreras-Cornejo et al., 2016). For example, the metabolic profile will be different for the same plant in the presence or absence of the pathogenic fungi or in the presence or absence of PGPF and in the presence of both PGPF and the pathogenic fungi (Contreras-Cornejo et al., 2016). Likewise, the metabolic profile for PGPF will be different in the presence or absence of host fungi and the host plant (Contreras-Cornejo et al., 2016). Similarly, the metabolic profile for the pathogenic fungi will be different in the presence or absence of the host plant (Contreras-Cornejo et al., 2016). The interactions between the host plant and the fungi are initiated by the detection of chemical signals that are released by the host plant in the rhizosphere (Brencic and Winans, 2005). These fungi move towards the host plant chemotactically (Brencic and Winans, 2005). Pathogenic fungi can enter the host roots directly by penetrating hypha and colonize the root cortex. *Fusarium* pp. and *Verticillium* pp. followed this mechanism (Yao and Allen, 2006). When these pathogenic fungi are able to reach the vascular system of the host plant they can easily spread all over the plant tissues (Lagopodi et al., 2002). These pathogenic fungi grow rapidly in the host and ultimately release a variety of mycotoxins like trichothecenes, fumonisins, and aflatoxin that either cause diseases in the host or kill the host (Lagopodi et al., 2002). For example, successful entry of *F. oxysporum* into the host plant leads to increased necrosis and ethylene-inducing peptide (Nep1) level that inhibit root and cotyledons growth and cause cell death (Bae et al., 2006). Mycotoxins produced by the pathogenic fungi are also phytotoxic in nature (Bacon and Nelson, 1994; Sharma et al., 2018). For example, *F. proliferatum* produces a large amount of fumonisins, a group of polyketide derived mycotoxins (Stępień et al., 2011). Biosynthesis of fumonisins is dependent on FUM1 gene expression and this gene expression increases when a suitable host is found by the fungi (Stępień et al., 2011). Hence the pathogenic fungi generally produce phytotoxins whose concentration increases when these fungi are inside the host.

When a plant is attacked by a pathogen, there are huge amount of metabolic changes within the host plant as compared to uninfected host plant (Singh et al., 2018). The change in plant metabolite profile is recognized by the accumulation of various low molecular weight antimicrobial metabolites and phytoalexins (Singh et al., 2018). These phytoalexins are specific depending upon the plant family and mainly include indoles, flavonoids, and terpenoids (Singh et al., 2018). After the interactions with the pathogenic fungi, the host plant either may be able to restrict the pathogen growth or may be able to restrict the disease development (Lagopodi et al., 2002). But the host plant when interacts with PGPF is able to activate defense mechanism without any actual infection. These PGPFs also help the host plant to develop a bio-control strategy against various pathogens (Vinale et al., 2008). For example, *T. virens* Gv 29-8 produces some peptaibols that have ISR elicitor effect and hence systematically induce defense in cucumber leaves (Viterbo et al., 2007) and *T. hamatum* accelerates defense mechanism in *Arabidopsis* plants against *Botrytis cinerea* (Mathys et al., 2012). Hence association between PGPF and the host plant is beneficial for both the partners as the host gets primed and PGPF get nutrient and shelter from the host.

When there are interactions between the pathogenic fungi and PGPF such as *Trichoderma* spp., then the result is different from the interactions with the host plant. Usually, there are many PGPFs that act as BCAs against these plant pathogens (Zeilinger et al., 2016). The bio-control activity of *Trichoderma* is due to mycoparasitism, antibiotic (toxin) production, secretion of enzymes especially chitinase and glucanases or any secondary metabolite, (Harman et al., 2004) and induction of defense response in the plants (Yedidia et al., 1999). These advantageous properties of *Trichoderma* spp. as BCA are due to two virtues of these fungi namely (a) presence of antibiotic producing genes and (b) the presence of transporter proteins (Kumari et al., 2015; Chaudhary et al., 2017). For example, a gene *tri5* in trichodermin biosynthetic pathway from *Trichoderma* spp. is involved in the production of the secondary metabolites-trichodermin and harzianum A, that help in the bio-control activity of the fungi (Vinale et al., 2009; Kumari et al., 2015). Moreover, the transporter proteins also play an important role in the bio-control activity of *Trichoderma* spp. (Chaudhary et al., 2017). These transporters efflux the toxins of other fungi produced in the vicinity (Ruocco et al., 2009). Due to these properties, *Trichoderma* spp. are able to overcome the static effect of other fungi and extreme competitive conditions (Benítez et al., 2004). This mechanism of mycoparasitism in *Trichoderma* spp. initiates with a chemotactic movement of the fungus towards the pathogenic fungi that is the host for *Trichoderma* spp. (Benítez et al., 2004). *Trichoderma* moves towards the host fungus and when its hypha comes in contact with the host fungus, lectins present on the host fungus mediate the attachment of cell wall carbohydrates of *Trichoderma* to the host (Inbar and Chet, 1995). After attachment to the hypha of *Trichoderma*, the hypha coils around the host fungus, forming appressorium-like structure to invade host fungus with the simultaneous release of CWDEs, proteases and secondary metabolites/toxins/antibiotics (Benítez et al., 2004). These low molecular weight biomolecules also play a very crucial

role in impeding the host defense mechanism (Howell, 2003). Hence, *Trichoderma* eventually kills the host fungi (Harman et al., 2004). This basic mycoparasitic mechanism is followed by almost every *Trichoderma* spp. known to process bio-control activities so far (Harman et al., 2004). It has been shown that in the presence of *Botrytis cinerea*, a change in the metabolic profile of *T. arundinaceum* was seen (Izquierdo-Bueno et al., 2018). It was observed that there is present a chemical cross-regulation between *B. cinerea* and *T. arundinaceum* where the sesquiterpenes of both fungi were found to play an important role in the biocontrol mechanism (Izquierdo-Bueno et al., 2018).

31.7 Conclusions and future directions

Human population is expected to doubly increase by 2050 as compared to the present rate of increase and naturally, the demand for food will also increase in the future. Since mankind depends upon various plants for the fulfillment of their requirements hence there is a need to increase the crop plants productivity. But most of the existing crop plants are susceptible to many plant pathogens, the fungal pathogens being the most potent threats to the crop plants all over the globe. The management of these harmful fungal pathogens require specific as well as broad range fungicides to be used in the crop fields. Because their management is necessary, hence various pathogen specific and broad range fungicides are commonly used in the crop fields. Prolonged use of these chemicals has a detrimental effect on the environment along with their adverse effect on human and animal health. Therefore, BCAs such as *Trichoderma* spp. could be an alternative to these chemicals for sustainable agricultural practices. Although many researchers have already discovered many things related to bio-control and plant growth promoting the activity of *Trichoderma* spp., there are still a number of probing queries need to be pondered over. In the present time we need to understand the bio-control and growth promoting mechanisms exhibited by *Trichoderma* strains to design more potent bio-control and plant growth promoting strains of this potent fungus. The enzymes of *Trichoderma* spp. involved in the bioremediation of soil are still unexplored. The enzymes responsible for degrading a wide range of insecticides can be studied for their better utilization in the bioremediation process. Hence a collaboration of genetic, molecular and biochemical based approaches is needed to understand and decipher new vistas in this sphere for the optimum use of this biocontrol agent on a sustainable basis.

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Applications of agriculturally important microorganisms for sustainable crop production

Hagera Dilnashin¹, Hareram Birla¹, Trinh Xuan Hoat², H.B. Singh³, S.P. Singh¹ and Chetan Keswani¹

¹Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India, ²Plant Protection Research Institute (PPRI), Vietnam Academy of Agricultural Sciences (VAAS), Ha Noi, Vietnam, ³Department of Mycology and Plant Pathology Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India

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32.1 Introduction

Sustainable agriculture is not only a technique but an extensive process based on a broad concept. It comprises of new developments in agricultural technology and management. Sustainable agriculture also highlights the inefficiency of conventional technique to meet the growing requirement of agricultural production with the increasing population in the 21st century (Ram et al., 2018). Conventional technique faces a major challenge in the form of reduced crop production, at higher cost. Repeated production on the same land reduces soil fertility, soil vitality, groundwater purity and beneficial microbe, insect life, leaving the crop plants vulnerable to parasites and pathogens (Keswani, 2015b). The primary issues are an ever increasing demand of fertilizers and pesticides, energy requirements for tilling to aerate soils and increasing irrigation costs. Conventional techniques offer high crop production and profits, only initially and thus, cannot be relied upon for future requirements. Moreover, the involvement of corporate in farming has led to detrimental effects on farming lands and surrounding environment, as it is only done for maximizing profit gains. It has also impacted the rural communities, which only depend upon agriculture. To maximize profit, cost cutting is done and the ones affected are the farm workers, due to which they struggle for basic living. This degrades the condition of rural communities and their contribution to the economy. Moreover, agricultural lands are often allotted to industries which reduce the available farm land and cause damage to the natural surroundings. Even the best farmlands face depletion due growing land requirement for residential and industrial construction. In economic terms, farming cannot compete with these growing needs. The earnings from the transformation of the farmland into housing subdivisions are astronomically greater than those achieved by any technique of farming (Singh et al., 2014).

Over the past century, the revolutions in the field of agriculture and technological developments have led to abundance of produced food for the ever increasing population. These developments have put up serious concerns which can become threat in the near future, if not addressed soon. It is evident that high crop production is required but the production should cause minimum damage to the nature. Thus there is a requirement of a unique way to achieve sustainable development while maintaining the natural ecosystem and biodiversity in its original form. Plant growth-promoting rhizobacteria (PGPR) offers a way which significantly reduces the degradation caused to the environment by chemical fertilizers. Kloepper and Schroth (1978) first described this word to define soil bacteria that colonize plant rhizosphere, growing in, on or around plant tissues that boost plant growth through multiple mechanisms. Researchers have been working extensively since then to understand and report the effects of these microorganisms. First, rhizobacteria screening for the *in vitro* production of phytohormones (Cassán et al., 2009; Rashedul et al., 2009; Abbasi et al., 2011) such as auxins (Khalid et al., 2004), siderophores (Filippi et al., 2011; Keswani et al., 2014; Singh et al., 2019), phosphorous solubilization or nitrogen-fixing (Peix et al., 2001; Fischer et al., 2007) was used to isolate PGPR from the rhizosphere and to screen for its growth-promoting activity in plants under axenic conditions (Singh et al., 2017). Once the PGPR applicant had showed promising performance under controlled circumstances, they were then used as inoculants for crops grown under natural circumstances in pots and/or field tests (Bisen et al., 2015; Keswani et al., 2016; Fraceto et al., 2018).

PGPR is majorly used to study the legume growth and development, specifically by means of nodulation and nitrogen fixation, limiting its use to rhizobia manipulation. Obviously, the primary reason for this is that a wide variety of soil-borne rhizobia species can develop leguminous symbiosis (Cooper, 2008). It is evident that rhizobia, a friendly plant associated bacteria and can be regarded as the most useful bio-fertilizer. Host-PGPR interaction benefits have been shown to include plant health and development, suppress microbial disease, and accelerate the accessibility and assimilation of nutrients (Mantelin and Touraine, 2004; Yang et al., 2009). PGPR has positive effects on plants by interacting directly with the host body and indirectly with the pathogens (antagonistic activity). Mechanisms involved in direct stimulation are: production of 1-aminocyclopropane-1-carboxylate (ACC)-deaminase to reduce the level of ethylene in developing plant roots. Mechanisms involved in direct stimulation are: production of 1-aminocyclopropane-1-carboxylate (ACC)-deaminase to reduce the level of ethylene in developing plant roots; Production of plant growth regulators such as auxins, gibberellins, cytokines and some volatiles; symbiotic nitrogen fixation; mineral solubilization such as phosphorus and other nutrients, etc. Indirect stimulation impacts biocontrol through antagonistic activity against phytopathogenic microorganisms that induce systemic reactions to plant resistance, interfere with bacterial quorum sensing (QS) devices, etc. Some reports indicate that PGPR may use more than one of these processes to improve plant growth (Bashan and Holguin, 1997; Ahmad et al., 2008). Recently an outstanding review has been released on these bacteria (Bhattacharyya and Jha, 2012). In some formulations that are commercially accessible, different PGPRs can be administered to plants (Lucy et al., 2004) and, lately, the popularity of microbial inoculants has risen significantly, facilitated by comprehensive and systematic studies that have improved efficacy and consistency (Thakore, 2006; Berg, 2009). There are three significant groups of microbial inoculants: arbuscular mycorrhizal fungi (AMF), PGPR, and symbiotic nitrogen-fixing rhizobia. Each group's beneficial capacity was explored individually (Dobbelaere et al., 2001; Barea et al., 2002; Verma et al., 2010; Murray, 2011).

In addition, countless trials are underway to assess the impacts of crop development by implementing various microbial combinations or consortia, such as AMF–PGPR, symbiotic nitrogen-fixing rhizobia (Singh and Kapoor, 1999; Swarnalakshmi et al., 2013). However, it is essential to understand the processes of promoting plant growth in order to decide which sort of microorganism is best to use with which plant in a specified scenario. Rhizosphere is the soil area, surrounding a plant root in which the roots influence the soil's biology and chemistry (Lugtenberg and Kamilova, 2009). Amino acids, organic acids, carbohydrates, sugars, mucilage and proteins are among the root exudates. Rhizosphere skill correlates with the capacity of rhizosphere to use organic acids as carbon sources. The plant species determine the structure of the rhizobacterial society just as variations in structure and quantities of root exudates most probably impact the microbial (Singh et al., 2016b; Bosco et al., 2017). Understanding the way in which plants choose the soil microbe for the microbial community of the rhizosphere is a significant scientific issue in the use of rhizobacteria as promoters for plant growth (Droge et al., 2012). Initially, the various mechanisms are highlighted, which are used by bacteria to improve plant growth and agronomic parameters. Followed by status of PGPR use on most important crops e.g. wheat, maize and rice, along with the ubiquitous legumes, primarily soyabean and dry bean varieties, is summarized in the review and discussed.

32.2 The contributions of soil micro-flora in sustainable agricultural production

There is a fundamental shift in agricultural practices and food production worldwide. The main driving force in the past was to increase the yield potential and productivity of food crops. At present, the desire is to combine sustainability to

the production. Sustainable agriculture aims at fulfilling human requirements while causing no damage to the natural environment and its resources. Optimal use and management of soil fertility and its physico-chemical properties can boost the sustainable agricultural production. Both rely on biodiversity of soil and soil processes. This implies management practices that enhance soil biological activity and build soil productivity and crop health on a long-term basis. Over the last 50 years, micro-organisms are being used to promote medical technologies, human and animal health, processing of foodstuffs, food safety and quality, and the genetic engineering, environmental protection, agricultural biotechnology and thus provide an improved solution for agriculture and municipal waste. Chemical and physical engineering alone cannot trigger ecological advances and even if they did, they need to address the practicality and economic challenges. Although microbial have helped develop various technologies in the agricultural field but the developed technologies are often not preferred as the ideal choice among scientists, as the results vary a lot. Microorganisms are only effective when adequate and optimal metabolism conditions are provided, including the available water, oxygen, pH and ambient temperature. Due to new technologies, the types of microbial cultures and inoculants on the market today have increased rapidly. In systems where technical guidance is coordinated with the marketing of microbial products, significant achievements are achieved (Keswani, 2015a; Singh et al., 2016a).

Microorganisms are widely used in agriculture as they significantly reduce the damage caused by the fertilizers and pesticides. Environmental pollution induced by excessive soil erosion and the related transportation to surface waters and groundwater of sediments, chemical fertilizers and pesticides and inadequate treatment of human and animal waste presents severe environmental and social issues globally. Although technicians have attempted to fix these issues using proven chemical and physical techniques, the use of microbial techniques and techniques was not possible. Microorganisms in the soil can communicate with the system of plants. Many microbes colonize the root and form biofilms or mantles and induce systemic resistance during the pest attack, many have the ability to solubilize or mobilize the growth-promoting substance produced by the mineral elements that make the plant healthy. The use of biofertilizers not only fixes atmospheric nitrogen, solubilizes mineral nutrients, but also helps to protect soil health, preserves soil fertility and crop quality.

32.3 Role of phosphate solubilizing microorganism in sustainable agriculture

Microorganisms are an important component of soil and by their beneficial or detrimental activities directly or indirectly influence soil health. Rhizospheric microorganisms mediate processes of soil such as decomposition, mobilization and mineralization of nutrients, storage of nutrients and water, fixation and denitrification of nitrogen. In addition, bacteria with the ability to solubilize phosphate can also transform insoluble phosphate compounds into soluble forms in soil and render them accessible to plants. Rhizospheric organisms' function in the solubilization of mineral phosphate was recognized. Extensive studies on the solubilization of mineral phosphate by naturally abundant rhizospheric microorganisms have been conducted since then. The *Bacillus* and *Pseudomonas* are key kinds of mineral phosphate solubilizers, while *oligospora* forms of *Aspergillus* and *Penicillium* have been tested *in vitro* and *in vivo* for their solubility of Tilemsi rock phosphate (Mali TRP), Kodjari rock phosphates (Burkina Faso, KRP), and Togolese rock phosphate (Senegal TRP) respectively. All 3 kinds of rocks by the fungus showed the ability to solubilize additional phosphate from *in vivo* rock phosphates (Duponnois et al., 2006). Most of the impact was directed towards understanding biological nitrogen fixation with the use of microorganisms as biofertilizers. On the other hand, important fungal genera are the fundamental work on phosphate solubilization by nodule bacteria (Motsara et al., 1995). Recently, *Arthrobotrys*, nematophagous fungus has been significantly less; although it is known that *Rhizobium*-legume symbiosis is the most limiting factor for nitrogen fixation.

Rhizobium phosphate solubilization is reported only a few times (Halder and Chakrabarty, 1993; Abd-Alla, 1994; Chabot et al., 1996) and *Azotobacter* (Kumar et al., 2001), a non-symbiotic nitrogen fixer. Microorganisms that solubilize phosphate are ubiquitous, the numbers of which vary from soil to soil. In soil, phosphate-solubilizing bacteria make up 1–50% of the total population and fungi 0.5–0.1%. The phosphate-solubilizing bacteria are usually 2–150 times greater than phosphate-solubilizing fungi (Kucey, 1987). The focused elevated percentage of PSM in the rhizosphere is known to be more metabolically active than isolated from sources other than the rhizosphere (Vazquez et al., 2000). In reverse, phosphate-solubilizing bacteria in the rhizoplane, the salt, pH and temperature-tolerant have been identified as maximum, followed by the rhizosphere and root-free soil in alkaline soils (Johri et al., 1999). The PSM strains with these strained characteristics should therefore serve as an outstanding model for studying the physiological, biochemical and molecular processes of phosphate solubilization in stressed ecosystems. It was also noted that, after repeated subculture, the phosphate-solubilizing bacteria lose their phosphate-solubilizing activity, but no phosphate-solubilizing fungal losses (Kucey, 1987). Phosphate solubilizing fungi usually generate more acids and therefore show more activity in

phosphate solubilization in solid and liquid media than in bacteria (Venkateswarlu et al., 1984). The capacity of PSM to solubilize phosphate also relies on the nature of the nitrogen source used in the media, with the existence of ammonium salts being more solubilized than when nitrate is used as a source of nitrogen. Proton extrusion was ascribed to the ammonium absorption compensation, which resulted in the decrease of extracellular pH (Roos and Luckner, 1984). In some cases, however, ammonium may lead to a decrease in phosphorus solubilization (Reyes et al., 1999).

32.4 Soil microbes for sustainable agriculture and environment

Overall, agriculture is the source of livelihood for farmers, high production rate and crop quality is mostly related to the requirement and profits earned from it. Thus, farmers must aim to protect the useful microorganisms and thus restore the natural environment. But this idea is not very popular among agriculture and natural farming supporters. The argument is that using organic changes to soils such as carbon sources, energy and nutrients will naturally boost helpful soil microorganisms. This can actually be true where organic materials are abundant for recycling only in small-scale farming. Soil microorganisms, however, are frequently used in a controlled way to overcome losses when pesticides are not used in repeated manufacturing. This describes why scientists have long been interested in using prospective and effective microorganisms as plant and soil inoculants, to alter the microbiological equilibrium that would improve soil quality and plant efficiency that are environment friendly. In agriculture, lower effectiveness of manufacturing is strongly related to bad energy conversion co-ordination that, in turn is affected by variables of plant physiology, climate, and other variables including soil-microbes. By producing bioactive metabolites, soil and rhizosphere microflora can accelerate plant growth and enhance their resistance to pathogens and harmful insects. Such microorganisms maintain plant growth and therefore have primary effects on the quality of soil and crops. Depending on their predominance and activity at any one time, a wide range of benefits are possible.

However, there is growing consensus that, without the use of artificial fertilizers, herbicides, insecticides and pesticides, it is possible to obtain maximum economic agronomic yield of high quality at higher net yields. Until recently, using conventional farming practices, this was not thought to be the very likely possibility. It is evident that sustainable agriculture positively affects the growth and population of soil micro flora, which directly improves the quality and production rate from agriculture. In particular, the very foundation of a future sustainable agriculture is healthy living soil with improved quality.

32.5 Agricultural productivity and plant growth promoting rhizobacteria

Plant growth promoting rhizobacteria includes the rhizosphere-colonizing N₂-fixing rhizobacteria, providing plants with nitrogen in addition to the well-characterized symbiosis of legume rhizobia. PGPR must colonize the rhizosphere around the roots, the rhizoplane (root surface) or the root itself (within root problem), regardless of the mechanism(s) of plant growth promotion. PGPR may influence plant growth either directly or indirectly. Direct pathway through PGPR involves either the provision of bacteria-synthesized compounds to support the uptake of multiple parameters of crop/fruit development, whereas in indirect pathway PGPR decreases or antagonizes the deleterious effects of one or more phytopathogens. The Bacilli and Pseudomonads are predominant among the various bacteria identified as PGPR (Podile and Kishore, 2007). PGPR directly affects plant growth through phytohormone production, inorganic phosphate solubilization, increased iron nutrition through iron-chelating siderophores, and volatile compounds affecting plant signaling pathways. In addition, through antibiotics, space and nutrient competition and induction of systemic resistance in plants against a wide range of root and foliar pathogens, PGPR reduces root pathogen and populations of other deleterious microorganisms in the rhizosphere, thus benefiting plant growth.

32.6 Plant growth promoting rhizobacteria in plant production

The world's next three first crops (million tons) are cereal maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticumaestivum* L.), according to the FAO after sugarcane. In 2011, global maize production was over 883 MT. In 2050, maize demand in developing countries is estimated to double (Rosegrant et al., 2009). Global rice production was nearly 723 MT in 2011. Rice has wide adaptability to various environments, making it the world's most widespread crop. It can grow in drought or shallow water conditions (up to 50 cm of water) and a wide range of latitudes and up to 3000 m of altitude. It is therefore considered by the FAO as a strategic crop for world food security. With regard to wheat, this cereal's global production in 2011 was nearly 704 MT. Wheat is a major renewable resource for food, feed and industrial raw material and the world's largest crop. While wheat has experienced a spectacular increase in yields

over the past century, annual yield increases have started to slow in 1995 and are now stagnating in almost every country (Reynolds et al., 2009). Since 1995, a clear connection has been built up between this stagnation and the increasing frequency of interfering climatic factors for example spring and drought during stem elongation, heat stress during flowering time and grain filling (Brisson et al., 2010; Lobell et al., 2011). In the same period, the world population increased from 5.8 billion to 6.6 billion and by 2050 is expected to exceed 9 billion. In the developing world, wheat demand is expected to rise by 60% by 2050, while climate change is expected to have a negative impact on production in the same areas by 29% (Dixon et al., 2009). In many countries, especially in developing countries, soybean is the most important legume included in human diet. In fact, in some regions of the world, soybean and soy products are the only food available to people (Bouchenak and Lamri-Senhadji, 2013). Soybean seeds are a concentrated source of isoflavones, making soybean unique among legumes. Moreover, soybeans are excellent source of high-grade protein as it contains low saturated fatty acids and high dietary fibers. In general, beans are particularly important in developing countries for human diets because they provide an important source of protein, vitamins (folate) and minerals (Ca, Cu, Fe, Mg, Mn, Zn) (Broughton et al., 2003). The nutritional benefit of beans in First World countries and their contribution to a healthy human diet that combats diseases such as cancer, diabetes, and heart disease are recognized by non-profit organizations targeting human food (Hangen and Bennink, 2003). Annual production of dry and snap bean, exceeds 21 MT, which is more than half of the total food production for legumes worldwide. Thus, use of PGPR as inoculants offers the potential alternative to sustainable development and production crops.

32.7 Role of cyanobacteria in agriculture and environmental sustainability

Cyanobacteria have adapted to survive in extreme conditions such as drought, salinity, low to high temperatures, etc. due to the evolutionary antiquity. Cyanobacteria were subjected to various stresses including salt stress and water main being, during phylogeny. Traditionally, cyanobacteria have been most adapted to drought and desiccation, probably owing to the existence of altered desiccation-resistant vegetative cells (spores or akinetes). Cyanobacteria are a continuously renewable biomass source which releases environmentally soluble organic substances as extracellular products also known as secondary metabolites that can be mineralized by microflora and thus benefit agricultural crops. For other organisms, including soil microflora, these substances may be the growth promoters and/or inhibitors (Zulpa et al., 2003). Reports on cyanobacteria that are available as biocontrol agents show that these organisms are efficient against pathogens in plants (Yuen et al., 1994). Although very little information on the impact of cyanobacteria and its metabolites on other plant, obtained from the field observations is available. In chronically contaminated environments (with heavy metals, salts, pesticides and other potential toxicants) tend to absorb and accumulate toxicants intracellularly. Level of pollutants in cyanobacterial cells can even be used as an indicator of pollution. Since cyanobacteria are the primary producers of various types of ecosystems, it is relevant from an ecological point of view tolerance of such organisms to various extreme environments (salinity, drought, contaminated soils, etc.). Mechanisms allowing cyanobacterial adaptations to such extreme conditions, however, are little known. In order to make sound predictions about the role of such microbes in restoring wastelands for sustainable agricultural development, more research is needed. Using successful indigenous cyanobacteria strains, as a prospective biofertilizer, not only enhances the physico-chemical and biological characteristics of the soil, but also contributes to the production of various agricultural crops such as wheat, rice, and pearl millet in contaminated agro-ecosystems. Regular use of cyanobacterial strains adapted to various challenging environment conditions appears promising for waste land management in ecologically sustainable way and improves nutrient status, mineralization of nutrients, soil microbial activities, soil stability, and crop growth.

An important nutritional element, phosphorous is mobilized by cyanobacterial biofertilizers through biological processes from an unusable to a usable form (Hegde et al., 1999). Cyanobacteria play an important role in different soil chemical transformations, thereby influencing the bioavailability of major nutrients (phosphorous) to plants. In order to increase crop production, cyanobacteria and PSB were used as biofertilizers (Singh et al., 1997; Earanna and Govindan, 2002).

In latest years, biofertilizers have appeared as promising parts of the integrated nutrient supply scheme of Indian agriculture. There were also reports of synergistic effects of effective soil microbes and cyanobacteria, organic acid excretions for increasing the availability of P, and a decrease in sulfide injury because of the increased O₂ level (Ordog, 1999). The efficiency of biotechnology instruments depends mainly on the cost and the simplicity of their operation and use. The manufacture and distribution of cyanobacterial biofertilizer to farmers is one of the applications arising from developing cyanobacterial biofertilizer technology. Instead of costly glass containers plastic bottles, polypropylene and polyethylene bags were used to disseminate liquid cyanobacterial plants. (Suresh et al., 1992). The key benefit of this technology is that farmers can generate the biofertilizer on their own with less extra inputs after obtaining the

soil-based starter cyanobacterial culture. Unfortunately, the technique of outdoor cyanobacterial production among the farming community is not so popular. The use of cyanobacterial biofertilizers is also limited due to lack of basic knowledge about the factors involved in establishing inoculated cyanobacterial species success and failure. Detailed field investigations on the development and application of efficient high-quality cyanobacterial soil inoculums in specific regions such as saline and drought-proven regions are also required. Thus, the use of bio-fertilizers is limited and there is a requirement for field application of cyanobacteria in agriculture.

32.8 Stability and productivity of desert soils

Dry soil (in desert and semi-arid areas) has significant limitations such as poor physical properties, low fertility and water scarcity (Nisha et al., 2007). The organic content in dry soil is biologically and chemically less stable and in arid areas tends to decrease very quickly, leading in poor soil quality. Generally, poor soil is associated with salinity, compaction, low organic carbon, and sodium, leading to reduction in water infiltration rates and aeration, thus increasing soil erodability, and the decreased number and biodiversity of micro-flora eventually adversely impacts plant growth and productivity. The quality of organically poor semi-arid soil has been significantly enhanced by the application of cyanobacteria improves the status of the soil's nitrogen, carbon, and other nutrients (Nisha et al., 2007). High organic carbon in treated soils could be attributed to the cyanobacteria's autotrophic nature, synthesizing and adding organic matter to the soil. Photoautotrophic and N_2 -fixing diazotrophic cyanobacteria enhance crop production by acting as natural biofertilizers by increasing soil status in both C and N. By incorporating organic matter into the soil, biogenic soil crusts consisting mainly of cyanobacteria appear to increase soil fertility (Belnap et al., 2001). In recent years, numerous studies have demonstrated that cyanobacterial crusts play an important role in semi-arid and arid ecosystems, together with a number of micronutrients, in order to increase soil stability and hydrology. (Orlovsky et al., 2004). Cyanobacterial inoculations were reported in disturbed soils to restore the nitrogen and carbon cycle population of microorganisms (Acea et al., 2003). Studies have discovered that cyanobacteria produce extracellular polymeric substances (EPS) that help to overcome water stress conditions and bind to soil particles. (Mazor et al., 1996). Because of its hygroscopic nature, cyanobacterial sheaths and EPS also play an important role in water retention (Decho, 1990). Therefore, the soil microflora adds to enhanced soil holding water ability (Verrecchia et al., 1995).

It has also been noted that cyanobacteria contribute to the improvement of soil nutrient status as carbon and nitrogen fixers in arid soils (Jeffries et al., 1992; Lange et al., 1994). Flaibani et al. (1989) reported that cyanobacteria-based exo-polysaccharides also contribute to desert soil recovery and improvement. Through the superficial network of trichomes/filaments, cyanobacteria appear to have a mechanical effect on soil particles as well as enmeshing with deep soil particles (Nisha et al., 2007). (Nisha et al., 2007). Some indigenous cyanobacterial strains in paddy and several other agricultural fields (Rogers and several other agricultural fields) have been noted to play a significant part in enhancing soil aggregation, soil holding capacity and soil aeration. Several studies have shown that cyanobacteria serve a very significant part in soil bioamelioration, also increase crop yields, not only through initiation of aggregation, but also by soil porosity protection, as the harmful effects of water supplementation are reduced (Marathe, 1972; Falchini et al., 1996). Diazotrophic cyanobacteria contribute significantly to the soil's fertility, particularly in paddy field under tropical conditions. However, most of these research have been carried out on paddy, and there is limited data regarding the potential role of bio-meliorant cyanobacteria in semi-arid regions that are not capable of growing paddy owing to restrictions on water supply. In semi-arid soils, native cyanobacterial strains have demonstrated remarkable potential for improving structural stability, nutrient status and soil productivity due to their intrinsic tolerance under restricted soil humidity (Manchanda and Kaushik, 2000; Nisha et al., 2007). The organic matter formed by 'Cyanocover' enhanced organic carbon of soil as well as water-stable soil aggregates (Malamissa et al., 2001). Better aggregation of soils can be ascribed to polysaccharides, generated by the blue green algae in cyanobacterial biofertilizers-treated soil (Ahmed, 1995).

According to the improved soil aggregation method, an increase in complete sugar content was discovered in soils with a cyanobacterial mat. Approximately 25% of their total biomass is also produced by the cyanobacterial consortium used as a biofertilizer (Nisha et al., 2007). In the upper soil layers, the activity of algal biomass and soil crust is focused, and EPS has a gluing effect on soil particles which leads to aggregation of soil. As stated by elevated soil enzymatic activity, cyanobacteria-producing EPS also appears to promote soil microflora activity. In turn, such microbial flora can produce more EPS, amplifying the effect further. Therefore, cyanobacteria play a very significant role in balancing C and N in the soil and were considered very important for the desert ecosystem. Removal of cyanobacteria

from arid sites decreases productivity and increases erosion of unprotected subsurface soils through wind and water exposure (Belnap and Gillette, 1997). Several workers reported that *Microcoleus vaginatus* formed algal crust and in extremely dry climates; *M. chthonoplasts* contribute significantly to soil stability (Campbell, 1979). Previous findings show that the drought resistant cyanobacterial biofertilizers strains application at a comparatively greater dose, enhances the physico-chemical and biological characteristics of the soil, also promotes crop yields under drought and water scarcity conditions. It is predicted that in desert and semi-arid soils, applications of cyanobacterial members are very important for the improvement of soil quality, microflora, nutrient status, mineralization of nutrient and crop growth in an ecologically sustainable manner. Understanding the drought resistant property of cyanobacteria can be helpful in sustainable farming and specificity of crops in drought. From the above points, it is obvious that under a stress system in the soil, indigenous cyanobacteria strains from semi-arid environments could be efficient. The remarkable tolerance of cyanobacterial strains to osmotic stress can enable them to succeed in semi-arid and arid soil agriculture with imminent water scarcity. Furthermore, Cyanobacterial strains adapted to elevated osmotic stress circumstances may be the suitable approach for improving crop productivity and reclamation of such wastelands for sustainable agriculture.

32.9 PGPR as biofertilizers

Azotobacter, *Enterobacter*, *Azospirillum*, *Rhizobium*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, and *Bacillus* strains are beneficial rhizobacteria, identified as PGPR (Rodriguez and Fraga, 1999). PGPR processes also enhance root development leading to a large-scale root system and enhanced root hair numbers (Mantelin and Touraine, 2004). Generally, large volumes of artificial fertilizers are used to replenish N and P in soil with the resulting elevated price and environmental risk. Most P is not available to plants in insoluble compounds. For crop plants, N₂-fixing and P-solubilizing bacteria (PSB) are vital as they increase N and P absorption and play a key role as PGPR in biofertilization (Zahir et al., 2004; Zaidi and Mohammad, 2006). Due to the production of plant growth-promoting substances, biofertilizers increases the availability of accumulated P (by solubilization), the efficiency of fixing biological N₂, and the availability of Fe, Zn, etc. (Kucey et al., 1989). N₂ fixation and PSB inoculation combination provide more balanced nutrition for agricultural plants such as sorghum, soybean, barley, blackgrams, and wheat (Alagawadi and Gaur, 1992; Abdalla and Omer, 2001; Tanwar et al., 2002; Galal, 2003; Belimov et al., 2005). Co-inoculation reports of rhizobium and PSB are rare and very little study has been undertaken on these guidelines, especially in India. In order to explore the impact of single and dual N₂-fixing and P-solubilizing bacterial species on the yields of distinct plants, extensive studies are therefore urgently required. Recent findings show that agronomic yields are considerably improved by PGPR inoculation therapy of arable soils (Harish et al., 2009; Yazdani et al., 2009). In nutrient-deficient calcisol soils, the PGPR strains like *Pseudomonas alcaligenes* PsA15, *Bacillus polymyxa* BcP26 and *Mycobacterium phlei* MbP18 had strong stimulating effects on plant growth and maize uptake (Egamberdiyeva, 2007). Phosphate mobilization, siderophores and antibiotics production, inhibition of ethylene synthesis and induction of plant systemic pathogens resistance has been enhanced due to the production of phytohormones (Han et al., 2004; Zahir et al., 2004; Ramazan et al., 2005; Zaidi and Mohammad, 2006; Turan et al., 2006). Recently, Kavino et al. (2010) disclosed that *P. fluorescens* strain, CHA0 in conjunction with enhanced production of chitin, nutrient content of leaf and perennial plant yields of banana crops indicate that PGPR may represent the prospective soil microflora to be applied for sustainability due to environmental issues created by excessive use and elevated fertilizer manufacturing expenses of fertilizers, PGPR may be the prospective soil microflora for sustainable and environmentally friendly agriculture to be implemented.

32.10 PGPR in agricultural saline soils

Soil salinity is a major concern for vegetables and other crops, since salinization suppresses plant growth, particularly in arid and semi-arid areas (Parida and Das, 2005). Changes in the physiology of plants facing salt stress have an adverse effect on nutritional uptake and crop growth as well. Because of their catabolic versatility, effective root-colonizing capacity and the ability to produce a variety of enzymes and metabolites, the soil-borne *Pseudomonas* received primary attention (Vessey, 2003). PGPR indirectly encourages crop development by reducing plant pathogens or directly by promoting nutrient uptake through phytohormone manufacturing (e.g., auxin, gibberellin, and cytokinin), enzymatically decreasing plant ethylene levels and/or producing siderophores (Kohler et al., 2006). Inoculations with AM fungi have been shown to improve the development of plants during salt stress (Cho et al., 2006). Kohler et al. (2006) demonstrated the beneficial effect on stability of soil aggregate strains of PGPR *Pseudomonas mendocina*.

Pseudomonas alcaligenes PsA15, *Bacillus Polymyxa* BcP26 and *Mycobacterium phlei* MbP 18 were all three of the isolates of the PGPR which were able to tolerate elevated temperatures and salinity to allow them to survive in arid and saline soils like calcisol (Egamberdiyeva, 2007). Salinity reduced the growth of lettuce irrespective of biological treatments and salt stress levels. *P. mendocina* inoculating crops have significantly more shoot biomass than controls and it is suggested that inoculation with chosen PGPR might provide a better way to relieve salinity stress in salt sensitive crops. There are possibly enormous expenses for improving soil salinity, although salinity has an effect on agriculture, biodiversity and the environment. Contributions from PGPR to plant health and its osmotolerance mechanisms are crucial. Since salt fields in agriculture rise every year worldwide, it is a serious problem (Paul and Nair, 2008). MSP-393 viz can detect osmotolerance processes. De novo synthesis of osmolytes and the overproduction of salt-stress protein efficiently nullify high-osmolarity damaging impacts. The perfect bioinoculant for the cultivation of the saline soil crops may be *Pseudomonas fluorescens* MSP-393 (Paul and Nair, 2008). Early stages remain for research into the interaction of PGPR with other microbes and their effects on the physiological response of plant crops under different soil salinity regimes. AM fungi could serve as a potential tool to alleviate salinity stress in salt-sensitive crops, especially inoculations with selected PGPR and other microbes. Thus, a comprehensive study is needed in this region, which can help to develop strategies to promote sustainable agriculture of saline soils by the use of PGPR and other micro-organisms, especially the AM fungus.

32.11 Conclusion

Sustainable agricultural system improves human health, provides economic and spiritual benefits to producers and consumers. It protects the environment and helps produce enough food for increasing food requirements with increasing world population. In most developing countries, irregular population growth and land demolishment lead to the enhanced food demand. Sustaining agricultural output through enhanced soil quality management is important for the food security and growing poverty alleviation. The optimal use of chemical nitrogen fertilizers is limited due to its high cost and inefficiency of most farmers to purchase it. This adversely affects the production of crops. In addition, a significant amount of urea N is lost due to various mechanisms including volatilization of ammonia, denitrification and loss of leaching, causing environmental pollution problems. This can be limited by using biological N₂-fixation technology. This technique reduces the use of urea N, prevents soil organic matter depletion and environmental pollution. Various bio-fertilizer systems, like PGPR and cyanobacteria, on a limited scale are employed especially in rice agro ecosystems. Further research is needed to determine their N supplement potential before large-scale extension of microbial biofertilizer systems at the farm level. Significant progress in the field of bacterial and cyanobacterial biofertilizer technology has been achieved worldwide, and it has potential to offer an effective and potential means to enrich soil fertility and enhance rice farming yield. However, as part of sustainable agricultural development programs, the technology needs further improvement for its better exploitation. Cyanobacteria and PGPR offer new genetic components and bioactive chemicals to biotechnologists with multi-fact use in agriculture and sustainability. In our understanding, current and future advances in formulation and application of PGPR and cyanobacteria diversity, colonization capacity, interaction mechanisms, could facilitate their development as the reliable components of sustainable farming systems management. In order to increase crop production and health, cyanobacteria and PGPR offer an environmentally sustainable approach. Molecular techniques improve our capacity to comprehend and manage the ecosystems of the rhizosphere and can enhance the efficacy of new products. Genetic improvement in colonization and efficacy of PGPR strains may require addition of one or more characteristics related to plant growth promotion. In order to improve the establishment and proliferation of useful microorganisms, host crop genetic manipulation for root-associated traits is being pursued. Health and safety testing is crucial so that non-target effects on other organisms including allergenicity, toxigenicity and pathogenicity, environmental persistence, and potential for horizontal gene transmission can be minimized.

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Beneficial microorganisms in the remediation of heavy metals

Nasser Delangiz¹, Mohammad Behrouzi Varjovi², Behnam Asgari Lajayer³ and Mansour Ghorbanpour⁴

¹Department of Plant Biotechnology and Breeding, Faculty of Agriculture, University of Tabriz, Tabriz, Iran., ²Department of Agronomy and Plant Breeding, Faculty of Agricultural and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran., ³Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran., ⁴Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran.

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33.1 Introduction

Heavy metals are one the most contaminating agents around world, therefore required emergency efficient action to remove them from environment (Asgari Lajayer et al., 2017a). Heavy metals are not degradable in the nature and easily can accumulate in the body of organisms so they can affect wide range of organisms in the nature from primitive microbes to progressive mammals negatively. However, they have huge significance in our economy and we cannot stop using them. Some of the metals, such as zinc (Zn), magnesium (Mg), Iron (Fe), copper (Cu), manganese (Mn) has different biological roles especially root and shoot growth. However, others known heavy toxic metals such as aluminum (Al), cadmium (Cd), lead (Pb), and mercury (Hg) do not have any known biological duties (Asgari Lajayer et al., 2019a).

Using microbes as remediators is a novel approach which is more compatible with environment in comparison with chemical and physical methods. In addition, it is inexpensive, highly efficient, and is not dependent on high-technology. Several microbial agents (bacteria, fungi, and microalgae) have been discovered as bioremediation approach till now. Using microbes directly or their enzymes or metabolic pathways individually, microbial fuel cell (MFC), and biofilm based bioremediation are some the most novel methods to bioremediate heavy metals (Igiri et al., 2018). Heavy metals removal occurs either through detoxification or remediation. Bioremediation of heavy metals involves transforming the more toxic form to less toxic ones which is occurred through living organism such as bacteria (Gautam et al., 2015). New methods for heavy metals bioremediation/bioremoval are introduced every day. New investigations broaden our understanding of microbes-metals interactions and the impact of heavy metals on the microbes and vice versa. Such studies can result in constructing novel and more efficient microbial systems for heavy metal removal from the nature (Asgari Lajayer et al., 2019a).

Chemical state of heavy metals, pH, humidity, temperature, the level of gases (such as O₂) and the composition of microbes in the medium are some factors which affect the efficiency of bioremediation (Igiri et al., 2018). The metal binding sites of heavy metals are saturated with H⁺ in acidic pH. In this condition, heavy metals tend to be free ions with high ability to absorb the negative charged surfaces. At lower pH, the high concentration of positive charge on the surface of the heavy metals inhibits the binding of them to adsorbent surfaces, therefore reduces the toxicity of heavy metals. Increase in temperature increases the bioavailability of heavy metals so the toxicity of them (Olaniran et al., 2013).

Genetic engineering can modify the architecture of pathways involved in heavy metals bioremediation so biotechnologist can deploy more efficient heavy metal bioremediator microbes.

33.2 Heavy metals' toxicity in the environment

Both natural and anthropogenic factors take part in environmental pollution. Among the natural factors are volcanic activities, forest fires, soil erosion, earth crust weathering. However, human activities like as urban sprawl spreads, forests degradation, and widespread industrial activities have played a more destructive role (Asgari Lajayer et al., 2018, 2019b). These factors in general, created a disaster condition for our mother Earth and all kinds of beings that had much better conditions before the industrial revolution.

Although, some metals are needed for creatures, with regard to the use of metals in different sectors, it is unlikely that they will be considered one of the most important pollutants in the world. Among the metals that have physiological function in organisms are iron, manganese, copper, cobalt, molybdenum and vanadium. Of course, high levels of these metals can also produce different levels of toxicity for different organisms (Ghorbanpour et al., 2016; Asgari Lajayer et al., 2017b). But some of metals do not have any biological function. These include arsenic, mercury, lead, chromium, cadmium and antimony (Asgari Lajayer et al., 2017a).

The negative effect of heavy metals on organisms is known as their toxicity is dependent on the bioavailability and absorption amount of them (Rasmussen et al., 2000) (Table 33.1). Physiology and biochemistry of organisms are changed under heavy metals stress. They can weaken organisms' defense system and their ability to remediate heavy metals. As they induce reactive oxygen species (ROS) generation in the cells. ROS can act as electron carrier in the cells and destructs cell components such as DNA, proteins, lipids and membrane (Giner-Lamia et al., 2014).

Heavy metals can also deactivate enzymes and disturb the vital chemical reactions inside cell. They change the configuration of enzymes through competitive and noncompetitive interactions (Gauthier, et al., 2014). The vital chemical groups of enzymes may be affected by heavy metals. For instance, Cr can interact with thiol and carboxyl group of enzymes and deactivate them (Cervantes et al., 2001). Also, they can affect the replication and transcription process of DNA through binding to phosphorous group of DNA. Therefore, they can induce the mutation in DNA structure and cause cancer (Cervantes et al., 2001). At high concentrations, these can cause skin rashes, stomach upset (ulcers), respiratory problems, weakening of the immune system, damage to the kidneys and liver, hypertension, alteration of genetic material, cancer, neurological disorders, and even death (Nava-Ruiz and Méndez-Armenta, 2011).

33.3 Microbes vs heavy metals

The toxicity of heavy metals, the time when the organism is exposed to that metal and the sensitivity of the organism to heavy metals are among the determinants of the toxicity of these metals for a specific organism (Table 33.1). When plants are exposed to heavy metals stress, their basic physiological processes such as photosynthesis, respiration and even cell division, get into trouble (Jadia and Fulekar, 2009; Pourrut et al., 2011). These metals can also disrupt the function of cytoplasmic enzymes through the formation of oxidative stress and disrupt cellular structures in plants (Chibuike and Obiora, 2014; Gaur et al., 2014). The destructive effects of these metals on human beings are also undeniable. In a study the effects of lead, cadmium and mercury toxicity concentrations on children's health have been studied (Hsueh et al., 2017). In this study, 89 children have been studied and found that the concentration of cadmium and lead in these blood children has a direct relationship with developmental delays.

Heavy metals have been existed in the geochemical cycles, therefore, it is expected the microbes are capable of interacting with them (Olaniran et al., 2013). However, in contaminated condition, the concentration of heavy metals is more than natural conditions. Bioremediation ability needs some tolerant microbes under such condition (Edwards and Kjellerup, 2013).

Microbial bioremediation is an innovative approach to remove and recover heavy metals from contaminated sites (Ayangbenro and Babalola, 2017). Bioremediation can be done by various microbes (bacteria, archaea, fungi, and algae) and plants. This method can be alternative for most of inefficient, expensive and polluting physical and chemical methods. Bioremediation can be used naturally or can be improved through adding nutrients, electron acceptors or other agents (Ayangbenro and Babalola, 2017). Biotransformation using exopolysaccharide (EPs) and enzymes, synthesis of metallothioneins, and exclusion are some of the important mechanisms adopted by microbes to survive in heavy metals contaminated environments (Dixit et al., 2015).

As microbes are exposed to heavy metal contamination, they develop some ingenious mechanisms to detoxify and resist excessive concentrations of them. The mechanisms involve redox state modification, precipitation, ion exchange,

TABLE 33.1 Negative effects of some of the most dangerous heavy metals on human and other organisms and some microbe capable of their bioremediation.

Metal	Contaminating resource	Harmful effects on organisms	Microbes	References
Chromium (Cr)	Mining, volcanic eruptions, rock weathering	In human: destructive for kidney, bones and liver. carcinogen. In other organisms: Its harmful effect is widespread, from vertebrates to plants and algae.	<i>Pseudomonas putida</i> <i>Bacillus</i> sp. strain GZ-22	(Park et al., 2000; Zhao et al., 2017)
Lead (Pb)	Fossil fuels specially gasoline, dyeing factories, batteries and faucets and toys.	In human: It has destructive effect on the brain especially in children. Immunotoxicity, reproductive system toxicity, hypertension, and anemia are other harmful effects on human. In other organisms: Shellfish are affected from even low levels of Pb. It also disturbs the body function of phytoplankton. It also has destructive on soil microorganisms.	Microbial Consortium	(Meril et al., 2016)
Mercury (Hg)	Fluorescent lamps, hospital waste, electrical industry.	In human: Hg can disturb children's development. Nerve, digestive and immune system of human are affected by Hg. In other organisms: Methyl mercury is highly toxic for birds' embryo. Pb accumulates in the body of fish and can poison the food chain easily.	<i>Pseudomonas putida</i> <i>Geobacter sulfurreducens</i>	(Zhang et al., 2012; Schaefer et al., 2011)
Arsenic (As)	Fuel burning, volcanic eruptions.	In human: As is highly toxic for human. It can affect our respiratory, nervous, reproductive, and lymphatic systems negatively. It also has destructive effects on our skin and blood. In other organisms: The effects are so wide from disturbing photosynthesis in plants, growth inhibition, behavioral effects, to even death in some sensitive organisms.	<i>Herminiimonas arsenicoxydans</i> <i>Thiomonas</i> sp. <i>Pseudomonas xanthomarina</i> S11	(Muller et al., 2007; Arsène-Ploetze et al., 2010; Koechler et al., 2015)
Copper (Cu)	Mining, Cu plumbing, electronic industries	In human: Vomiting, dizziness, stomachache and headache (in low concentrations). Kidney and liver damage and even death (in high concentrations). In other organisms: Cu can accumulate in both plants and animals. Plants survive hardly in Cu enriched soils. It is also damaging for earthworms and soil born microorganisms.	<i>E. coli</i> <i>Kocuria flava</i> <i>Sporosarcina koreensis</i>	(Djoko et al., 2010; Cornu et al., 2017)
Cadmium (Cd)	Zinc smelting, waste batteries, e-waste, paint sludge, incinerations and fuel combustion, painting industries, batteries, burning fuels.	In human: Cd can cause flu like symptoms (fever, muscle pain), in high concentrations can result in cancer. In other organisms: Cd is toxic for all kind organisms (plants, animals, and microbes). It also can cause kidney malfunction in birds and mammals. It can easily accumulate in vertebrates' body.	<i>Pseudomonas aeruginosa</i> <i>Rhodobacter sphaeroides</i> sp.	(Chellaiiah, 2018; Peng et al., 2018)

(Continued)

TABLE 33.1 (Continued)

Metal	Contaminating resource	Harmful effects on organisms	Microbes	References
Molybdenum (Mo)	Spent catalyst, coal combustion.	In human: Mo has negative effect on fetal development. It can also damage reproductive system. In other organisms: Mo can inhibit growth in various organisms. Also, it may have destructive effects on kidney, reproductive system. Diarrhea is the other negative effects of Mo on them.	<i>Bacillus</i> sp. Strain A.rzi	(Othman et al., 2013)

surface complexation, electrostatic interaction (Yang et al., 2015). Also, oxidation and methylation are two major biochemical reactions to alleviate the toxicity of heavy metals (Ramasamy and Banu, 2007). Microbes can change the oxidation level of heavy metals so increase their negative effects. Metals can be consumed by microbes as electron donors to release energy from food sources. They take part in anaerobic respiration (Barkay et al., 2003).

Bioavailability, heavy metals concentration, humidity, O₂, pH, redox potential, temperature, and medium structure are some of the environmental factors which affect the efficiency of microbial bioremediation heavy metals (Mani and Kumar, 2014). Methylation is the other important biological reaction which plays important role in heavy metal bioremediation. Bacteria from *Clostridium*, *Pseudomonas*, *Escherichia*, and *Bacillus* methylate Hg (II) and generate methyl mercury, a gaseous compound. Also, microbial methylation of selenium (Se), arsenic (As), and Pb results in the production of dimethyl selenide, arsines, dimethyl lead respectively (Ramasamy and Banu, 2007). Microbes also accumulate heavy metals intrinsically through complexation with various components. This process is said intracellular sequestration. For instance, glutathione sequesters Cd in *Rhizobium leguminosarum* l (Lima et al., 2006). And this happens in Cadmium-tolerant *P. putida* strain through cysteine-rich low molecular weight proteins (Higham et al., 1986).

Fungi are strong microbes to remove heavy metals from the nature as their cell walls consist of chitin, nitrogen-containing polysaccharide, proteins, lipids, polyphosphates, and mineral ions which all can bind with the heavy metals. Indeed, the exterior part of fungal cell walls as act as a ligand to trap the heavy metals (Shipra et al., 2011; Selvam et al., 2013; Gupta et al., 2015; Xie et al., 2016). Lipids and polysaccharides are two components of fungal cell walls sites act as heavy metals ligand. Among these ligands, amine groups (NH₂) are the strongest binding agents.

33.4 The role of biofilm in heavy metals removal

Biofilm is organized as stable matrix which may contain bacteria, and some eukaryotes such as fungi, microalgae (Costerton, 1995). Human can benefit from the bioremediating capacity of biofilms and change the contaminating industries to cleaner and safer ones (Karn et al., 2017). The investigation of biofilm remediation capacity in bioremediating of the oil spill on the shorelines of Prince William Sound, Alaska, back in 1989 proved its potential in the remediation of highly polluted sites (Atlas and Hazen, 2011; Karn et al., 2017). The role of biofilm in the industry and healthcare was thoroughly studied. However its role in the bioremediation especially heavy metals bioremediation was not studies as well (Karn et al., 2017). Biofilms can act as biostabilization agent in the nature. Therefore, they can trap the heavy metals. There are some reports of biofilm direct role in their bioremediation (He et al., 2018; Liu et al., 2018; Ma et al., 2018; Mallick et al., 2018). Biofilm is so resistance to high concentration of heavy metals. For instance, the efficiency of *Rhodotorula mucilaginosa* was estimated about 4.79–10.25% for planktonic cell and 91.71–95.39% for biofilm (Grujić et al., 2017). Biofilms contain some emulsifier or surfactant components which can bind with heavy metals and trap them in an isolated condition (El-Masry et al., 2004) and they can act as trapping agent for the insoluble heavy metals (Fomina and Gadd, 2014).

33.5 The mechanisms of microbial detoxification of heavy metals

Bioremediation of heavy metals is unique as biological processes cannot completely remove them from the nature (Edwards and Kjellerup, 2013). Understanding the mechanism of heavy metals detoxification can lead experts to find more efficient microbes to remove them from the environment. Microbial detoxification of heavy metals can be done

through changing in valence state, vacuole compartmentalization, metal binding, and volatilization. Different metals have different valence states and different toxicity levels. For instance, methylmercury, which is highly toxic, can be converted to Hg (II), which is much less toxic, by mercury resistant microbes. This process is mediated by organomercurial lyase (Wu et al., 2010). Phytochelatins, metal binding peptides, and metallothionein are some of the metal binding factors.

In volatilization process, microbes convert metals to volatile state. This process can be done about Hg (II) and Se as they are the only heavy metals which have volatile forms. Hg (II) can be volatilized by mercury resistant microbes and transformed to Hg (0). This reduction process is mediated by MerA enzyme (Wu et al., 2010). Also, Se (V) can be reduced to Se (0). The removal of Se (V) by filamentous fungi was thoroughly described by (Siddiquee et al., 2015).

Microbes also employ different methods to remove and detoxify heavy metals in contaminated regions. Biosorption, bioaccumulation, biomineralization and biotransformation are these methods (Lin and Lin, 2005). These methods can be taken by both living and dead microbial bodies. Heavy metal removal through living organisms is more complicated than through dead organisms. Living organisms need a suitable environment (food source, temperature, humidity, and etc) to be lived. So, using them as bioremediator agents can be more expensive. Exploiting microbes to detoxify heavy metal regions, experts should consider the level of their resistance and remediation capacity under contaminated condition (Sharma et al., 2000).

Metals can be absorbed by the binding sites present are present in the surface of microbes' cell walls (Malik, 2004). This kind of heavy metals trapping is passive and independent from the cellular metabolisms. In the other biosorption process metals can pass through the cell wall of the microbe. This process is active and directly depends on the cellular metabolisms so the energy consumption. The accumulator microbe should be tolerant to heavy metal(s) is (are) accumulated and should be able to convert them to less toxic form(s) (Fomina and Gadd, 2014; Mosa et al., 2016).

Microbes are negatively charged on their surfaces as they contain anionic groups (such as alcohol, amine, carboxyl, ester, hydroxyl, sulfhydryl, phosphoryl, sulfonate, thioether, and thiol groups). So, they can bind to metal cations (Gavrilescu, 2004). Microbes can also secrete metal binding agents such as polymers (polysaccharides and proteins) to outside of their cells. This way they can trap heavy metals in the outside of their cells and isolate heavy metals from the sensitive organisms in the nature.

33.6 Conclusions

The ability of microbes to remediate/remove heavy metals promises novel efficient system to get rid of their toxic. The biofilm components (polysaccharides peptidoglycans) are natural heavy metal biosorbents. This is a cost-effective technique and can be applied in broad environmental conditions. This chapter provides an opportunity to reveal the role of microbial cell, biofilm, and their metabolites towards remediation of heavy metals and environmental research. Future studies can lead scientists to achieve a highly efficient and eco friendly microbial-based method to remove destructive heavy metals in the environment.

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MOLECULAR ASPECTS OF PLANT BENEFICIAL MICROBES IN AGRICULTURE

Understand the role of microbes in varied ecosystems as well as their advanced biotechnological applications

Edited by

Vivek Sharma, University Centre for Research and Development, Chandigarh University, Gharuan, India

Richa Salwan, College of Horticulture and Forestry (Dr.Y. S. Parmar University of Horticulture & Forestry) Neri, Hamirpur, India

Laith Khalil Tawfeeq Al-Ani, Department of Plant Protection, College of Agriculture, University of Baghdad, Baghdad, Iraq

Key Features

- Covers the molecular attributes of biocontrol, Trichoderma, nematophagous, PGPR, and mycorrhizal associations involved in the three-way interaction between beneficial microbes–hosts–pathogens
- The importance of cross-protection and induction of the plant defense and resistance
- Highlights the role of technological interventions in exploring the molecular mechanisms
- Provides detailed and comprehensive insights on recent trends in use of genetic engineering of microbes for agricultural application

Molecular Aspects of Plant Beneficial Microbes in Agriculture explores the diverse interactions of plant microbes with their host, highlighting the complex, dynamic, and adaptive relationships. This book highlights diverse types of interaction including the pathogenic and symbiotic relationship which can lead to either a decrease or an increase in crop productivity.

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